Comparison of Initiation of Protein Synthesis in Procaryotes, Eucaryotes, and Organelles

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INTRODUCTION

Ribosome-mediated synthesis of proteins, using messenger ribonucleic acid (mRNA) as the template, is one of the hallmarks of earthly life forms. It is not surprising that the cellular machinery for protein synthesis has been highly conserved. Ribosomes, from bacteria to mammals, have the same overall structure. The processes of aminoacyl-transfer RNA (tRNA) binding, peptide bond formation, and ribosome translocation are virtually identical in all organisms, and all ribosomes display the same division of labor between the small and large sub-units. It comes as a surprise, therefore, that in the initiation process—those steps preceding formation of the first peptide bond—there are striking dissimilarities between procaryotes and eucaryotes. Although current information about the translation system in organelles is not sufficient to reveal the workings of the initiation process, there are hints that chloroplasts follow the bacterial mechanism. What little we know about the structure of mitochondrial ribosomes and mRNA, however, seems incompatible with either the procaryotic or the eucaryotic initiation mechanism. Thus, the problem of how a ribosome recognizes the correct site in mRNA for synthesis of the first peptide bond seems to have

been solved not just once during evolution, but several times, and in several different ways.

What follows is, first, a brief structural comparison of the machinery involved in translation, with emphasis on the components peculiar to initiation. Second, the structures of mRNAs from procaryotes, eucaryotes, and organelles are described. Finally, I attempt to define and compare the mechanisms by which ribosomes from various sources recognize the correct initiation sites in mRNA. Many important aspects of ribosome structure and function which bear only peripherally on the initiation process are alluded to in the following pages without extensive discussion. Additional details may be found in excellent reviews by Hershey (180) and Nierhaus (329). Throughout the text, references to procaryotes or to bacteria pertain only to eubacteria. Archaebacteria, which constitute the second branch of the procaryotic kingdom, are discussed only peripherally, and in such cases they are mentioned by name. *Eucaryotic* refers to 80S ribosomes present in the cytoplasm and encoded entirely in the nucleus of eucaryotic cells. Ribosomes present in the organelles of eucaryotic cells are always referred to by naming the organelle—either mitochondrion or chloroplast.

THE TRANSLATIONAL MACHINERY

Structural Components of Ribosomes

Ribosomal proteins. The protein components of *Escherichia coli* ribosomes have been studied exhaustively. There are 21 protein species in the small ribosomal subunit and 32 in the large one (329). Since protein S20 from the small subunit is identical to protein L26 from the large subunit, the 70S *E. coli* ribosome contains 52 different protein species. Most of the proteins are present at one copy per ribosome. The only exception is the large acidic protein, designated L7/L12, of which four copies are present. Antibodies have been used to map the positions of many of the proteins on the surface of the ribosome (463). The primary sequences of all 52 proteins from *E. coli* have been determined (50, 463, 530), and the entire set has been dissembled and successfully reassembled into functional ribosomes (8, 310, 333, 381). These marvelous exploits carried out with ribosomes from *E. coli* have not yet been extended to other organisms. Preliminary investigations of the ribosomal proteins of gram-positive bacteria suggest that they correspond closely to those of *E. coli*, except for the absence of protein S1 (184).

The gross anatomy of eucaryotic ribosomes as revealed by electron microscopy resembles that of bacterial ribosomes (44), except that eucaryotic ribosomes are bigger (Table 1). Fractionation by two-dimensional polyacrylamide gel electrophoresis reveals a much larger set of proteins in eucaryotic cytoplasmic ribosomes than is found in bacteria—somewhere between 70 and 80, rather than 52. Among eucaryotic organisms, there is some structural variation in the ribosomal proteins (118, 372), but this does not seem to reflect functional divergence, since ribosomes from organisms as distant as yeasts and mammals are functionally interchangeable in vitro (495, 518). Most eucaryotic ribosomal proteins, like those from bacteria, are small, basic, and insoluble (534). However, the two dimensional electrophoresis pattern of ribosomal proteins from eucaryotes shows no similarity to that of procaryotes. Immunological studies have revealed shared determinants on a few procaryotic and eucaryotic ribosomal proteins (118, 481, 534), but cross-reactions are not common. One of the striking exceptions to the

TABLE 1. Sizes and protein compositions of ribosomes from various sources

Source	Sedimentation coefficients			No. of protein species		
	Small subunit	Large sub-unit	Complete ribosome	Small subunit	Large sub-unit	Refer-ence(s)
E. coli	30S	50S	70S	21	32	329
Chloroplasts of higher plants	30S	50S	70S	22–24	32–35	61, 108
Mitochondria						
Yeasts	37S	50S	70–74S	33	38	113
Plants	40S	60S	78–80S			
Mammals	~30S	~40S	55–60S	33	52	295
Eucaryotic cytoplasm						
Yeasts	40S	60S	80S	30–32	41–44	113, 252
Rodents	40S	60S	80S	~31	~49	534

general absence of homology concerns the acidic protein L7/L12 (in the bacterial nomenclature), which mediates the guanosine 5'-triphosphate (GTP)-dependent steps of protein synthesis. Not only do antibodies against bacterial L7/L12 inhibit the function of eucaryotic ribosomes (191), but also yeast ribosomes stripped of their own acidic proteins can be functionally reconstituted by the addition of L7/L12 from *E. coli* (396). The highly conserved character of that protein has been confirmed by direct sequence analysis (270). Homology between components of procaryotic and eucaryotic ribosomes was also demonstrated by the ability of *E. coli* protein L1 to bind to *Dictyostelium* 26S ribosomal RNA (rRNA) in a way that resembles the binding of L1 to *E. coli* 23S rRNA (153). Thus, select features have been conserved between the ribosomal proteins of procaryotes and eucaryotes, although the nonhomologies outweigh the homologies. The arrangements and the expression of genes encoding the ribosomal proteins are also dissimilar in the two systems. In *E. coli*, the ribosomal protein genes are clustered into several operons (334), each of which gives rise to a polycistronic mRNA which is autoregulated at the translational level by one of the ribosomal proteins (335, 540). It seems unlikely that this remarkable regulatory scheme is repeated in eucaryotes, since the genes encoding eucaryotic ribosomal proteins are not closely linked (122, 535) and the mRNAs appear to be monocistronic (122, 164). There are hints of coordinate regulation of ribosomal protein synthesis in eucaryotes (131, 150, 350), but the mechanism remains unknown. The ribosomal protein genes are reiterated approximately 10 to 20 times in the genome of higher eucaryotes (312). They are present at one or two copies per haploid genome in *Saccharomyces cerevisiae* (122) and at only one copy in *E. coli* (334).

Chloroplast ribosomes resemble those of procaryotes and differ markedly from eucaryotic cytoplasmic ribosomes. Bacterial and chloroplast ribosomes are similar in size (Table 1), topography (494), RNA components (described below), and protein composition (61). Although the individual protein components of chloroplast ribosomes have not been studied in detail, immunological cross-reactivity has been shown between *Chlamydomonas* chloroplast ribosomes and those of *E. coli* (407). At least 11 of the chloroplast ribosomal proteins are encoded within the organelle (108, 121); the rest are presumably encoded in the nucleus.

The greatest difficulty in extending this discussion to mitochondria is that the structure of mitochondrial ribosomes cannot be generalized. Mitochondrial ribosomes range in size from smaller than bacterial ribosomes to larger than eucaryotic cytoplasmic ribosomes (Table 1) (56). A striking and perplexing characteristic of all mitochondrial ribosomes is their high content of protein relative to RNA. The protein content of mitochondrial ribosomes ranges around 70%, versus 50% for eucaryotic ribosomes and 37% for *E. coli* ribosomes. The nature of mitochondrial ribosomal proteins, at least as judged by electrophoretic mobility, varies tremendously even between closely related organisms. Only one ribosomal protein appears to be encoded within the mitochondrial genome in *Neurospora* (258); all the rest are encoded in the nucleus and must be imported into the organelle. No immunological cross-reactivity has been detected between proteins from the large ribosomal subunit of mitochondria and proteins from bacterial or eucaryotic ribosomes (359).

The biochemical characteristics described above suggest that procaryotic and chloroplast ribosomes are closely related; eucaryotic ribosomes show some similarities to those of bacteria but also show many important differences, and mitochondrial ribosomes are so unique that they cannot be classified readily. These conclusions are supported by the results of subunit exchange experiments. Functional ribosomes can be reconstituted by mixing ribosomal subunits from *E. coli* and chloroplasts, but not by mixing mitochondrial ribosomal subunits with those from *E. coli* or chloroplasts (159, 261). Patterns of susceptibility to antibiotics confirm the close relationship between bacterial and chloroplast ribosomes, as distinct from eucaryotic ribosomes. Although mitochondrial ribosomes are susceptible to most inhibitors of bacterial protein synthesis, the concentrations required for inhibition often differ markedly (45). The "mixed" susceptibility of archaebacteria to inhibitors of protein synthesis (105a, 228, 351) raises questions about the popular view that ribosomal inhibitors are either procaryote or eucaryote specific. Thus, the pattern of susceptibility to inhibitors is probably less reliable than structural parameters for classifying ribosomes.

Ribosomal ribonucleic acids (rRNAs). Bacterial ribosomes invariably contain three RNA components, designated 5S, 16S, and 23S. The larger eucaryotic ribosomes generally contain four RNA species: 5S, 5.8S, 18S, and 25–28S. Other variations are occasionally seen in eucaryotes, such as the splitting of the largest RNA into two pieces in insects (430), the cleavage of 5.8S rRNA into two fragments in *Drosophila* (348), and the presence of extra low-molecular-weight RNAs in some protozoa (405), but these deviations are rare. The RNA components of chloroplast ribosomes are strikingly similar to those of bacteria, except that the 3'-terminal sequence of bacterial 23S rRNA occurs as a separate 4.5S

TABLE 2. RNA components of ribosomes from various sources[a]

Source	16S rRNA and its homologs in the small ribosomal subunit	RNAs in the large ribosomal subunit			
		23S rRNA and its homologs	5S rRNA	5.8S rRNA	Other
Bacterium: E. coli	16S, 1,541 nucleotides (52)	23S, 2,904 nucleotides (51)	+ (110)	—[b]	—
Chloroplasts					
Chlamydomonas	16S	*23S[c]	+		3S, 7S (380)
Higher plants	16S, ~1,490 nucleotides (413, 489)	23S, ~2,900 nucleotides (103, 478)	+(477)		4.5S (476)
Mitochondria:					
Yeasts and fungi	15S, 1,686 nucleotides (269)	*21–23S, 2,865 nucleotides (234)	—	—	—
Higher plants	18S	26S	+ (445)	—	—
Mammals	12–13S, 954 nucleotides (109)	16S, 1,559 nucleotides (109)	—	—	—
Eucaryotic cytoplasm:					
Yeasts	18S, 1,789 nucleotides (385)	25–26S, 3,393 nucleotides (508)	+ (110)	+ (110)	—
Higher animals	18S, 1,825 nucleotides (273, 393)	28S	+ (110)	+ (169)	—

[a] References are given in parentheses for those rRNAs for which the entire nucleotide sequence has been determined, either by direct analysis of the RNA or by sequencing the corresponding gene.

[b] —, This rRNA species is absent from the organism or organelle in question.

[c] Asterisk indicates that the gene encoding the largest rRNA species may be interrupted by an intervening sequence. This is a highly variable feature. For example, introns occur in the mitochondrial large rRNA in some yeast strains, but not in others (41). Introns rarely if ever occur in the (functional) nucleus-encoded rRNA genes of higher eucaryotes, although they are found in Physarum and Tetrahymena (58, 528).

RNA species in some chloroplasts. Mitochondria display the greatest structural diversity, both in the sizes of the major RNA components and in the variable presence of the 5S rRNA species. Table 2 lists the RNA components found in mitochondria from a few sources; the full variety is described in reference 56. Table 2 also summarizes the subunit distributions of the various rRNAs, indicates their exact sizes, and provides references for those rRNAs that have been sequenced. The bewildering variety of low-molecular-weight RNAs associated with the large ribosomal subunit might be explained by fragmentation of the largest RNA species. For example, although E. coli ribosomes contain neither the 5.8S rRNA characteristic of eucaryotic ribosomes nor the 4.5S rRNA found in most chloroplast ribosomes, sequences homologous to both 5.8S and 4.5S rRNAs are present in E. coli 23S rRNA. Figure 1 illustrates the postulated relationship between bacterial 23S rRNA and some of the low-molecular-weight rRNAs from nonbacterial sources.

The organization of rRNA genes and their modes of transcription can be summarized briefly by referring to Fig. 1. In all cases except yeast mitochondria, the small-subunit RNA (16S–18S) and large-subunit RNA (23S–28S) are cotranscribed, with the small-subunit RNA positioned closer to the promoter. In bacteria, 5S rRNA is cotranscribed with 16S and 23S rRNAs. There are 7 copies of the 16S-23S-5S transcription unit in the E. coli genome (334) and 9 or 10 copies in the genome of Bacillus subtilis (280). In chloroplasts, the 16S-23S-(4.5S)-5S rRNA genes are linked, as in bacteria, and tRNAs are encoded in the spacer region between the 16S and 23S rRNAs, as in bacteria (see legend to Fig. 1); but, surprisingly, chloroplast 5S rRNA is transcribed from an independent promoter (171, 476). The chloroplast genome contains two sets of rRNAs except in Euglena, where there are three sets (45). In the nucleus of eucaryotic cells, 5S rRNA is separately transcribed—indeed, by a polymerase distinct from that which transcribes the linked 18S-5.8S-28S genes. In lower eucaryotes, the 5S rRNA component maps near the other rRNA genes and the whole unit may be tandemly repeated, but 5S rRNA genes are not linked to other rRNA genes in higher eucaryotes. The rRNA genes are repeated several hundredfold in the genomes of most eucaryotes. Detailed information about the arrangement and amplification of eucaryotic rRNA genes is provided in a thorough review by Long and Dawid (279). The complexities of rRNA transcription and processing have also been covered in recent reviews (45, 129, 353).

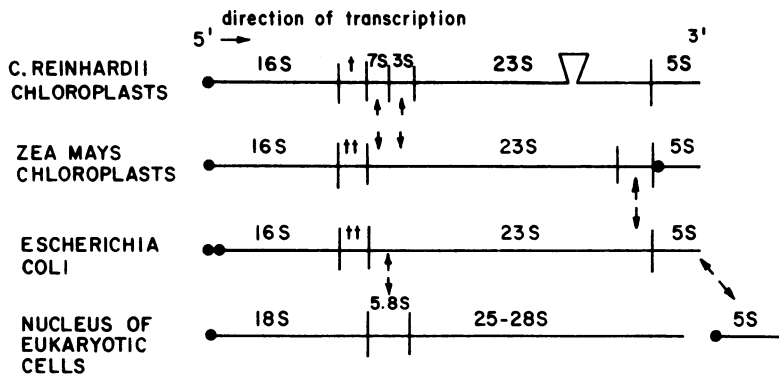Homology among the high-molecular-weight rRNAs from various sources is evident in both

FIG. 1. Schematic representation of rRNA transcription units, showing the relationships among some of the rRNA species. ● indicates the position of a transcriptional promoter. Sequence homologies which bear on the origins of the small rRNAs are indicated by vertical arrows. The 5.8S rRNA from eucaryotes shares about 50% homology with the 5' end of *E. coli* 23S rRNA (202, 324). The 5' end of eucaryotic 28S rRNA is homologous not to the exact 5' end of *E. coli* 23S rRNA but rather to a sequence that begins 158 nucleotides from the 5' end of 23S rRNA (325, 514). The 3' end of *E. coli* 23S rRNA is 65% homologous to the 4.5S RNA species present in the large ribosomal subunit of plant chloroplasts (286, 288). The 4.5S RNA is not found in *Chlamydomonas reinhardii* chloroplasts, but two other small RNAs from *C. reinhardii* chloroplasts (7S and 3S) map between the 16S and 23S rRNA genes. The 7S and 3S rRNAs are homologous to the 5' end of 23S rRNA from maize chloroplasts and bacteria (380). For simplicity, the mature rRNA species are shown contiguous to one another. In fact, they are separated by spacer sequences of variable length which form part of the primary transcript but are eliminated during processing. Two tRNA genes (designated tt) form part of the spacer region of the rRNA operons in bacteria (280, 334) and chloroplasts (233). tRNAs are not encoded in the spacer region between 18S and 28S rRNA in vertebrates (169). In the mitochondrial genome of mammals (not shown), a single tRNA sequence comprises the entire spacer between the large and small rRNA sequences (11, 109). Yeast mitochondria are unique in that the small (15S) and large (21S) rRNAs are widely separated on the genome and are separately transcribed (266).

their primary and their secondary structures. The primary sequence homology between *E. coli* and chloroplast rRNAs is striking: 74% for the 16S species (413, 489), and 67 to 71% for the 23S species (103, 478). These data strongly support the proposed procaryotic ancestry of chloroplasts. Comparison of *E. coli* 16S rRNA with 18S rRNA from the small subunit of eucaryotic ribosomes reveals a much lower level of overall homology (393), although sequences in a few scattered regions are highly conserved (257). The most extensive homology between procaryotes and eucaryotes involves nucleotides 9 to 51, near the 3' end of *E. coli* 16S rRNA (166, 394, 500). Interestingly, the conserved region stops just short of the 3'-terminal eight nucleotides of *E. coli* 16S rRNA, which play such a crucial role in initiation. (A detailed comparison of the 3'-terminal sequences of small-subunit rRNAs is presented below.) The 12S–15S rRNA from the small subunit of mitochondrial ribosomes is sufficiently homologous to some regions of bacterial 16S and eucaryotic 18S rRNAs to argue that all derived from a distant common ancestor (109, 257, 269), but the striking homology observed between chloroplast and bacterial 16S rRNAs clearly does not extend to mitochondria. Ac-

cording to one set of criteria, the primary sequences of small-subunit rRNAs from present-day procaryotes and eucaryotes are closer to each other than either is to 12S rRNA from mammalian mitochondria (109). The sequence of small-subunit rRNA from yeast mitochondria is considerably closer than the corresponding mammalian mitochondrial species to *E. coli* 16S rRNA (257, 461), but the divergence between yeast mitochondrial and *E. coli* rRNAs is still remarkable. In contrast, analysis of the T_1 oligonucleotides derived from wheat mitochondrial small-subunit (18S) rRNA revealed striking similarity to bacterial 16S rRNA (37). This was confirmed by direct sequencing of the 3'-terminal 100 nucleotides of wheat mitochondrial 18S rRNA (406). If one acknowledges the rather clear link between the small-subunit rRNAs of plant mitochondria and bacteria and if one accepts that all mitochondria derive from a common ancestor, then one must concede a phylogenetic link between mammalian mitochondrial and bacterial ribosomes, despite the very limited sequence homology that persists today. (Arguments in favor of a monophyletic origin for mitochondria have been presented elsewhere [155]. The issue seems far from settled. Perhaps

the strongest evidence is that all mitochondria studied to date use a deviant genetic code [20, 38], although [unfortunately for the argument] the nonstandard codon assignments are not identical in all mitochondrial systems.) The commonality of small-subunit rRNAs is reinforced by the finding that all can be fitted to the same secondary structure model (461). Even the 12S–15S rRNAs from mammalian and yeast mitochondria, which appear highly deviant in terms of their primary structures, can be folded into a secondary structure that preserves most of the "universal" base-pairing interactions (11, 257, 269, 461). The proposed secondary structure models are intriguing, although few of them, apart from that of *E. coli* 16S rRNA (332), are supported by experimental evidence.

Sequence data and secondary structure models for 5S rRNA confirm the view that chloroplast ribosomes are very closely related to those of bacteria (100, 477), whereas eucaryotic cytoplasmic ribosomes are more distantly related (110). Neither eucaryotic nor chloroplastic 5S rRNA can substitute for bacterial 5S rRNA in reconstituting ribosomal subunits, however (24). When assessed in terms of the structural features that distinguish procaryotic 5S rRNA from eucaryotic 5S rRNA, wheat mitochondrial 5S rRNA displays some characteristics of both types, as well as several unique features (445). As noted in Table 2, 5S rRNA seems to be absent from the mitochondrial ribosomes of yeasts and mammals—a rather surprising deficiency in view of the important functions attributed to 5S rRNA in other ribosomes (110).

Regarding the main objective of this article, which is to compare initiation mechanisms, what hints emerge from this brief survey of ribosome structure? The confusing data about mitochondrial rRNAs might be viewed in either of two ways: since some mitochondrial ribosomes (notably, those from plants) are structurally quite close to those of bacteria, the possibility of functional homologies between mitochondria and bacteria should be considered; the alternative view is to emphasize that mitochondrial ribosomes from most sources are structurally quite divergent from procaryotic ribosomes at the present time. Thus, irrespective of the ongoing debate about their origin (156), it is not unreasonable to consider that mitochondrial ribosomes use a unique mechanism for initiation. In short, one should proceed with an open mind about mitochondria, a willingness to believe that some functions mediated by eucaryotic cytoplasmic ribosomes might differ from those of procaryotes, and a strong prejudice that the structural similarities between chloroplast and bacterial ribosomes portend functional similarities.

Soluble Protein Factors Required for Initiation

The so-called factors required for initiation differ from ribosomal structural proteins in that the factors cycle on and off the ribosome. Most of the initiation factors bind transiently to the small ribosomal subunit and, in one way or another, mediate formation of a complex between the small subunit, mRNA, and methionyl (Met)-tRNA$_i^{Met}$. (The conventional representations for initiator tRNA species from procaryotes and eucaryotes are tRNA$_f^{Met}$ and tRNA$_i^{Met}$, respectively. For convenience, however, I will use tRNA$_i^{Met}$ as a generic representation for initiator tRNA^{Met} from any source.) The initiation factors are released before or during joining of the large ribosomal subunit. The subsequent "elongation" phase of polypeptide synthesis is mediated by another set of protein factors. The brief discussion that follows will focus on the initiation factors.

Three highly purified proteins from *E. coli*, designated IF-1 (initiation factor 1), IF-2, and IF-3, mediate formation of initiation complexes with bacterial ribosomes in vitro. The initiation factors that function with eucaryotic ribosomes are far more complex. Eight factors were initially characterized (27, 491) and designated eIF-1, eIF-2, eIF-3, eIF-4A, eIF-4B, eIF-4C, eIF-4D, and eIF-5, where "e" stands for "eucaryotic." There are three recent additions to the list: a factor that mediates recycling of eIF-2 (432; A. Konieczny and B. Safer, J. Biol. Chem., in press), a factor called eIF-6 that promotes dissociation of ribosomal subunits (387, 497), and a protein that interacts with the 7-methylguanosine (m^{7}G) cap on mRNA (441, 442). The so-called cap-binding protein copurifies in some cases with eIF-3 and eIF-4B, which complicates the task of sorting out their respective functions. Additional stimulatory proteins have been purified from extracts of eucaryotic cells (179, 290) although their functional significance is not yet clear. Recent reviews by Hershey (180, 181) and Maitra et al. (290) summarize the biochemical properties of the traditional initiation factors and provide insights into their functions. The factors purified from rabbit reticulocyte lysates have been studied most exhaustively and are used herein to exemplify those of eucaryotes. To the limited extent that factors from other eucaryotic systems have been studied, they appear generally similar to reticulocytes.

Table 3 lists the partial reactions that are dependent on or stimulated by the recognized initiation factors. *E. coli* factor IF-2 and reticulocyte factor eIF-2 mediate analogous functions, namely, GTP-dependent binding of tRNA$_i^{Met}$ to the small ribosomal subunit. The exact mechanism of that reaction in the *E. coli* system is

unclear. In the absence of ribosomes, IF-2 forms a binary complex with formylmethionyl (fMet)-tRNA$_i^{Met}$ (354, 503), but that complex is unstable in vitro. Since IF-2 binds stably to 30S ribosomal subunits in the absence of Met-tRNA and GTP (111), the physiological interaction between IF-2, fMet-tRNA$_i^{Met}$, and GTP may take place on the surface of the bacterial ribosome. This contrasts with reticulocyte eIF-2, which forms a stable ternary complex with Met-tRNA$_i^{Met}$ and GTP in solution (25); the ternary complex then binds to the ribosome. In this respect, eIF-2 resembles the elongation factors, which mediate binding of non-initiator tRNAs via formation of ternary complexes with GTP. Curiously, reticulocyte eIF-2 also resembles elongation factors and again differs from procaryotic IF-2 in that an additional protein is required to recycle eIF-2 after its release from the ribosome as a complex with guanosine 5'-diphosphate (432; Konieczny and Safer, in press). The eIF-2 recycling factor has no counterpart in E. coli, since guanosine 5'-diphosphate does not remain bound to the bacterial factor IF-2 after hydrolysis of GTP. To summarize, factors IF-2 and eIF-2 function analogously in mediating the GTP-dependent binding of tRNA$_i^{Met}$ to ribosomes, although some details of the reaction mechanism differ in the two systems. In both procaryotes and eucaryotes, (e)IF-2 is a target for translational regulation (74a, 203a; G. Owens, L. Jen-Jacobson, F. Ruscetti, and L. Jacobson, submitted for publication). Several other initiation factors from procaryotes and eucaryotes mediate functions which are superficially similar (Table 3), but the underlying mechanisms may differ. For example, the equilibrium between ribosomes and their subunits is shifted, in favor of subunit formation, by binding of initiation factor(s). In E. coli this is accom-

TABLE 3. Functions mediated by procaryotic and eucaryotic initiation factors

Function	Initiation factor[a]	
	E. coli	Mammalian
Promotes dissociation or slows reassociation of ribosomal subunits	*IF-3 (68, 215, 471) IF-1 (89, 502)	*eIF-6[b] (497) eIF-4C (152)
Met-tRNA binding to the small ribosomal subunit	IF-2 (92)	eIF-2 (27, 491)
Recycling of (e)IF-2 after the large subunit joins and GTP is hydrolyzed	IF-1[c] promotes release of IF-2 from 70S ribosomes (26, 466)	Recycling factor mediates exchange of GDP for GTP after release of eIF-2 · GDP from the ribosome (432; Konieczny and Safer, in press)
mRNA binding to the small ribosomal subunit	IF-3 (389, 509)	eIF-1 (486) *eIF-3 ⎤ eIF-4A ⎬ (27, 491) eIF-4B ⎦ Cap-binding protein (441, 442)
Joining of the large ribosomal subunit	No factor required in E. coli	*eIF-5[d] (27, 355, 491) eIF-4C (355)
Other		eIF-4D enhances the puromycin reactivity of Met-tRNA (27)

[a] Where several factors are listed for a given function, an asterisk indicates the one that is quantitatively most important.

[b] The ability to prevent reassociation of ribosomal subunits had originally been attributed to the high-molecular-weight complex eIF-3 from mammalian systems (212, 487, 492). Recent studies suggest that a low-molecular-weight protein, designated eIF-6, has nearly all of the anti-association activity in both mammalian (497) and wheat germ (387) cell-free extracts. It is possible that eIF-6 associates with eIF-3 during the early stages of purification, thus explaining the earlier results.

[c] At an earlier step, IF-1 stabilizes the binding of IF-2 to 30S subuits (466). Thus, its role in initiation is far more complex than indicated in the table.

[d] This factor does not directly promote joining of the 60S ribosomal subunit. Rather, eIF-5 mediates GTP-dependent release of eIF-2 and eIF-3 from the 40S initiation complex (356), and the unencumbered 40S complex then rapidly associates with a 60S subunit.

plished by the binding of IF-3 to the small ribosomal subunit, whereas in eucaryotes eIF-6 binds to the large ribosomal subunit, thereby preventing joining of the large and small subunits (387, 497). Since it is not understood how either factor accomplishes its anti-association effect, however, the homology between procaryotes and eucaryotes might be more (or less) than present evidence suggests. Similarly, although it is known that five factors (Table 3) stimulate binding of eucaryotic mRNA to reticulocyte ribosomes in vitro, the mechanism of that crucial reaction (which requires only one factor in *E. coli*!) is not understood well enough to assess the similarities between procaryotes and eucaryotes.

Notwithstanding that disclaimer, a brief discussion of the initiation factors that mediate mRNA binding seems in order. It is important to know whether initiation factors are actively involved in selecting initiation sites in mRNA or whether they merely stabilize binding at sites selected by the small ribosomal subunit. Most of the evidence from procaryotic systems indicates that recognition of initiation sites in mRNA is an intrinsic property of the ribosome, as first revealed by Lodish's mixing experiments with ribosomes and factors from *E. coli* and *Bacillus stearothermophilus* (274, 275; see discussion below). In support of that conclusion, studies using washed (i.e., factor-free) ribosomes from *E. coli* revealed that the low level of binding obtained in the absence of IF-3 occurs at the authentic initiation sites in coliphage mRNAs (458, 553). Nevertheless, bacterial initiation factors—particularly IF-3—influence the relative utilization of initiation sites by differentially stabilizing the binding of ribosomes to mRNAs. With bacteriophage R17 mRNA, for example, addition of initiation factors to washed *E. coli* ribosomes increases ribosome binding to the beginning of the coat protein cistron much more than they enhance binding to the A-protein cistron (458). The situation in eucaryotes superficially resembles that in *E. coli* in that translation of some mRNAs is preferentially stimulated by certain initiation factors. The ability to discriminate among mRNAs has been variously attributed to eIF-4A, eIF-4B, and a component associated with eIF-3 (130, 147, 213), but definitive identification requires more highly purified factors. The molecular basis underlying the apparent discrimination by eucaryotic factors is not known. Some reasonable guesses have been made, however, about the mechanism of discrimination by initiation factors from procaryotes (454, 458).

We do not know whether, under physiological conditions, mRNA encounters initiation factors in solution (forming a complex which then binds to the ribosome) or whether mRNA encounters initiation factors only on the surface of the small ribosomal subunit. Several of the factors bind to both free ribosomal subunits and mRNA, but it has been difficult to evaluate the functional significance of the latter complexes. Bacterial component IF-3, for example, binds to phage mRNA (209), but the preformed complex of IF-3 and coliphage MS2 RNA was inert when subsequently tested for translation in the presence of anti-IF-3 antibodies (553). Several eucaryotic initiation factors also bind to mRNA, including eIF-2 (216) and eIF-3 (182, 512); however, neither of those interactions is specific for mRNA (70, 181, 512). Recent studies revealed that reticulocyte factors eIF-4A and eIF-4B bind to mRNA, as assayed by retention of labeled mRNA on a nitrocellulose filter (158, 418). That interaction is nonspecific in that the factors also bind to rRNA and tRNA, but mRNA seems to work best (W. Merrick, personal communication). Binding of eIF-4A and eIF-4B to mRNA is likely to be functionally significant, since it is at least partially inhibited by cap analogs (158, 343, 418) and is strikingly dependent on adenosine 5'-triphosphate (ATP) hydrolysis (158). The latter observation is exciting in view of the long-recognized ATP requirement for initiation by eucaryotic ribosomes. The purified 24,000-dalton cap-binding protein also binds (i.e., can be cross-linked to) capped mRNAs (441). Although these studies demonstrate that initiation factors from eucaryotes can bind to mRNA in the absence of ribosomes, it is not yet clear that the soluble mRNA-protein complexes are functional intermediates in initiation. A recent theoretical analysis predicts a protein factor that escorts mRNA onto the ribosome (141a), but predictions do not constitute proof.

In both procaryotes and eucaryotes, the initiation factor requirements for binding of natural mRNAs are more complex than those for binding of the triplet adenylyl-uridylyl-guanosine (AUG). Certain synthetic oligonucleotides, however, appear to be closer analogs of mRNA, since their binding depends on the complete set of factors (204). It is curious that, with both procaryotic and eucaryotic ribosomes, the requirement for some initiation factors (as well as one ribosomal protein!) is abolished when denatured mRNA is used as the template (31, 440, 505, 509). This has prompted the suggestion that some initiation factors function by denaturing mRNA, but that idea awaits further study.

At the present time, one can only speculate that there probably are some proteins that serve as translational initiation factors in mitochondria and chloroplasts. None has yet been identified. In vitro assays with *Euglena* chloroplast ribosomes and poly(adenylate, uridylate, guanylate) [poly(A,U,G)] as a template are stimulated by *E.*

coli factor IF-2 (L. Spremulli, personal communication), auguring a similar factor in chloroplasts. Analysis of organellar elongation factors is somewhat more advanced than the study of their initiation factors. Chloroplast elongation factors are functionally exchangeable with bacterial elongation factors, but not with eucaryotic cytoplasmic factors (154). In one study, elongation factors from bacteria did not support translation of poly(U) by mitochondrial ribosomes (496); but other reports differ (56). At least one of the elongation factors is encoded within the chloroplast genome (447, 519). Mitochondrial elongation factors, however, appear to be nucleus encoded (377).

Initiator Transfer RNAs

A distinctive feature of bacterial, chloroplast, and mitochondrial translation systems is that $tRNA_i^{Met}$ is formylated. Transformylase activity (catalyzing transfer of formate from N^{10}-formyltetrahydrofolic acid to give N-fMet-tRNA) was detected years ago in extracts from E. coli (1), mitochondria (167, 283, 436), and chloroplasts (411). Amino acid incorporation in isolated mitochondria and chloroplasts is strongly depressed by N^5-formyltetrahydrofolate, a competitive inhibitor of formylation reactions in which N^{10}-formyltetrahydrofolate serves as the donor (281). Surprisingly, in vivo studies revealed that bacteria can initiate protein synthesis under conditions that preclude formylation of Met-tRNA (172). But this ability correlates with (and presumably requires) a structural change in the tRNA (21a, 395). Presumably, wild-type bacterial $Met-tRNA_i^{Met}$ works in vivo only when it is formylated, as is generally observed in vitro (256). The formylation requirement is due, at least in part, to discrimination by E. coli initiation factor IF-2, which interacts only with tRNAs that carry an N-blocked amino acid (473). $fMet-tRNA_i^{Met}$ is reserved for initiation in bacteria, chloroplasts, and mitochondria; each contains a second species (designated $Met-tRNA_m^{Met}$) that donates its methionine to internal positions in the polypeptide chain (293). The cytoplasm of eucaryotic cells also contains two distinct Met-tRNAs, one of which functions in initiation and the other of which functions in elongation (190, 437), but the initiator tRNA in eucaryotes is not formylated. As indicated below, eucaryotic $Met-tRNA_i^{Met}$ can be formylated artificially by incubation with the transformylase from E. coli. In contrast to its positive effect in procaryotes, formylation of eucaryotic $Met-tRNA_i^{Met}$ reduces its affinity for the cognate initiation factors (373). It is interesting to note, parenthetically, that archaebacteria also initiate with an unformylated species of Met-tRNA (526).

The unique role of $Met-tRNA_i^{Met}$ in protein synthesis implies that it has a structure different from all other tRNA species. Nucleotide sequences have been determined for initiator tRNAs from bacteria, chloroplasts, mitochondria, and a wide variety of eucaryotic cells. From the sampling of sequences shown in Fig. 2, one can search for structural features which identify initiator tRNAs, using the following guidelines: initiation factors, either alone or in combination with the ribosome, must recognize some feature(s) in $Met-tRNA_i^{Met}$ which are absent from non-initiator tRNA species in the same organism; at least some of the identifying features should be common among initiator tRNAs, since heterologous $Met-tRNA_i^{Met}$ species can be substituted for the homologous species with some success (Table 4); but the inefficiency of such heterologous reactions (see footnotes to Table 4) suggests that some critical features may be unique to each class of initiator tRNAs. The only universal feature revealed by comparison of the primary sequences in Fig. 2 is the occurrence of four guanine · cytosine (G · C) base pairs in identical positions in all initiator tRNAs (57); these conserved residues are circled in the figure. In several initiator tRNA species, the anticodon loop appears to have an unusual conformation, as revealed by its sensitivity to S1 nuclease (536) and by X-ray diffraction analysis (533). But the generality of that important finding remains to be established. Initiator tRNAs from higher eucaryotes are distinguished as a class by three features: the presence of AUCG or AΨCG (Ψ indicates pseudouridine and C indicates cytidine) instead of the usual sequence TΨC^G_A (T indicates ribothymidine) in positions 54 to 57 of loop IV, an A instead of the usual pyrimidine in position 60, and a C instead of the invariant U in position 33, adjacent to the anticodon. (Initiator tRNAs from lower eucaryotes are identical to those of multicellular eucaryotes in the first two features, but differ in the third; i.e., U rather than C is found in position 33 in $Met-tRNA_i^{Met}$ species from yeasts [536], Tetrahymena [254], and Scenedesmus [338].) Procaryotic initiator tRNAs lack the three distinguishing characteristics of eucaryotic initiator species, but have their own distinctive structures. All procaryotic initiator tRNAs studied to date have an unpaired nucleotide at the 5′ terminus and an unmodified A residue on the 3′ side of the anticodon. The hypermodified A_{37} residue found in many other tRNAs is believed to restrict flexibility in codon-anticodon pairing (336). Thus, the unmodified A_{37} residue in bacterial $tRNA_i^{Met}$ might account for its ability to respond not only to AUG but also to GUG and other codons. Since the most striking characteristic of procaryotic $tRNA_i^{Met}$ is the unpaired
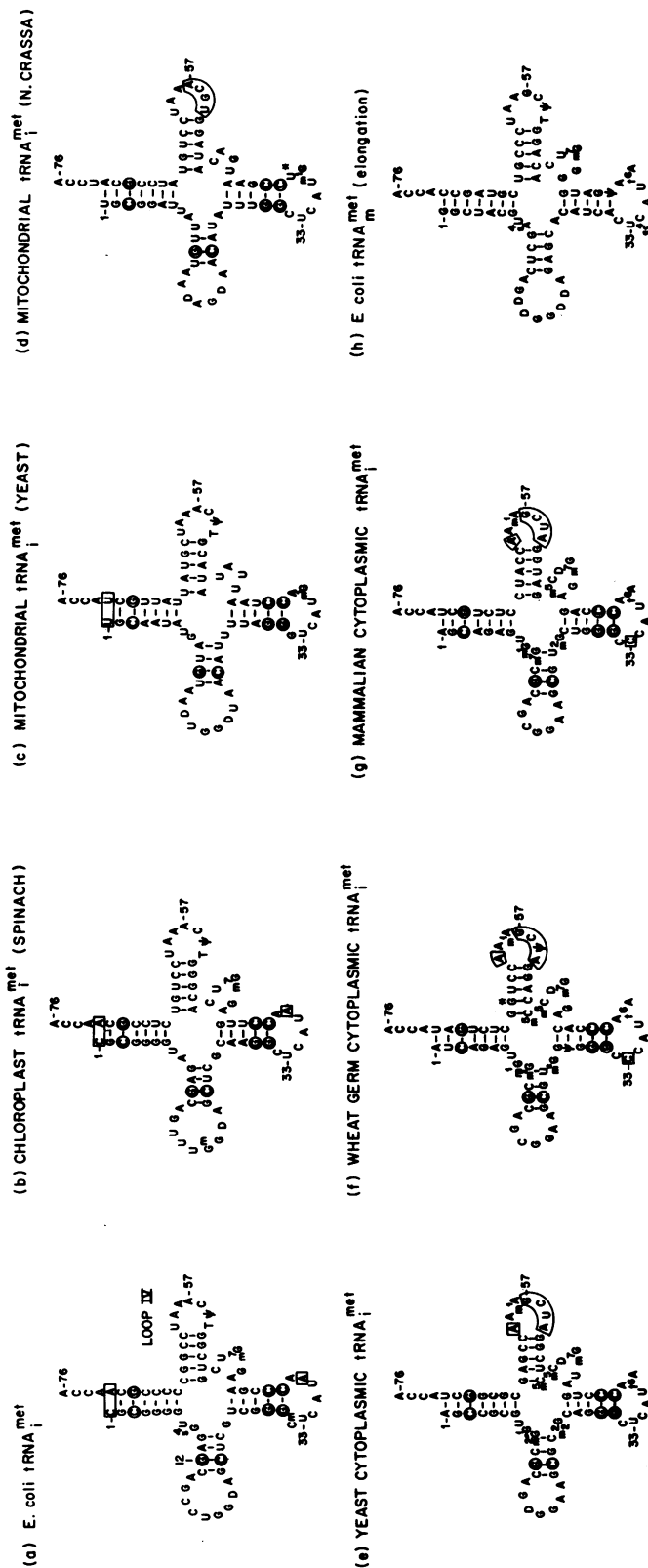
FIG. 2. Nucleotide sequences of seven initiator tRNAs, compared with non- initiator Met-tRNA$_m$^{Met} from *E. coli*. The encircled nucleotides are common to all initiator tRNAs that have been sequenced, except for some archaebacterial species (not shown) which lack the G$_{12}$ · C$_{23}$ pair (253). Features distinctive to initiator tRNAs from a given class are boxed. Numbering begins at the 5' end of the RNA and follows the system of Gauss et al. (127). The anticodon (CAU) occurs in the loop at the bottom of each structure. Unknown derivatives are marked with an asterisk. The sequences are reproduced from the following references: *E. coli* tRNA$_i$^{Met} and tRNA$_m$^{Met} and yeast and mammalian cytoplasmic initiator tRNAs from reference 536, spinach chloroplast tRNA$_i$^{Met} from reference 57, yeast mitochondrial tRNA$_i$^{Met} from reference 59, mitochondrial tRNA$_i$^{Met} from *N. crassa* from reference 174, and wheat germ cytoplasmic tRNA$_i$^{Met} from reference 133. Nucleoside modifications are indicated as follows: s^4, 4-thio-; m, methyl-; m$_2^2$, N^2,N^2-dimethyl-; t^6, N^6-threoninocarbonyl; ac^4, N^4-acetyl-. D, 5,6-Dihydrouridine.

TABLE 4. Ability of tRNA$_i$^{Met} species to function with proteins from homologous and heterologous sources[a]

Protein(s) and source	Functional properties of tRNA$_i$^{Met} from:					
	E. coli	Chloro-plasts	Mitochon-dria	Yeast cytoplasm	Wheat germ cytoplasm	Mammalian cytoplasm
E. coli						
Synthetase	+	+[b]	+[b]	+	+	+
Transformylase	+	+[b]	+ or −[c]	+	−[d]	+
Ribosomes and factors	+		+[e]	+[f]	+[d]	+[g]
Eucaryotes						
Synthetase	+		−[b]	+	+	+
Ribosomes and factors	±[h]		n.t.[i]	+	+	+

[a] Some of the data, as well as the general design of the table, are from an excellent review by Ofengand (336).
[b] See reference 163.
[c] Although mitochondrial initiator tRNA from *N. crassa* can be formylated by the *E. coli* enzyme (174), the closely related tRNA$_i$^{Met} from yeast mitochondria cannot be formylated (59).
[d] In heterologous systems with *E. coli* or rabbit reticulocyte ribosomes, Met-tRNA$_i$^{Met} from wheat embryos does function, but it tends to donate methionine into internal positions of the polypeptide chain (134).
[e] See reference 174.
[f] Provided that the yeast Met-tRNA$_i$^{Met} has been formylated, it is fully functional in protein synthesis carried out in vitro with *E. coli* ribosomes and coliphage f2 mRNA (479).
[g] See references 63.
[h] Ranu and Wool (373) found that *E. coli* Met-tRNA$_i$^{Met}, whether formylated or not, bound only weakly to eucaryotic initiation factors. In other studies, Met-tRNA$_i$^{Met} from *E. coli* functioned efficiently in partial reactions with eucaryotic factors and ribosomes, but methionine donated by the procaryotic tRNA either was not incorporated efficiently into peptide linkage (106) or was incorporated into internal positions of the polypeptide chain (376).
[i] n.t., Not tested.

nucleotide at the 5' end, it was surprising to find that that feature is not critical for function. The ability of *E. coli* tRNA$_i$^{Met} to bind to ribosomes in vitro was not lost when the 5'-terminal C was converted to U (472). Nonetheless, an unpaired 5'-terminal nucleotide is diagnostic of procaryotic initiator tRNA species. Chloroplast initiator tRNAs share this distinctive feature (Fig. 2). Indeed, the overall degree of primary sequence homology between bacterial and chloroplast initiator tRNAs (81 to 84% [57]) is nearly as high as the sequence conservation among procaryotic initiator tRNAs as a group (84 to 97% [513]). In the case of mitochondrial initiator tRNAs, it is once again difficult to generalize. tRNA$_i$^{Met} from yeast mitochondria resembles bacterial and chloroplast initiator tRNAs in having an unpaired 5'-terminal nucleotide and the sequence TΨCA in loop IV (Fig. 2). But mitochondrial tRNA$_i$^{Met} from *Neurospora crassa* lacks both of those features. The most striking feature in *N. crassa* mitochondrial tRNA$_i$^{Met} is presence of UGCA instead of the usual TΨC^G_A in loop IV. This is somewhat reminiscent of eucaryotic initiators, although the sequence that has been substituted for TΨC^G_A in mitochondrial tRNA$_i$^{Met} is different from that in eucaryotes. The structural divergence between mitochondrial initiator tRNAs from two closely related organisms (*S.*

cerevisiae and *Neurospora*) stands in striking contrast to the high degree of sequence conservation among procaryotic initiator tRNAs as a group.

In addition to wondering what features in tRNA$_i$^{Met} are recognized by the ribosome-associated machinery, it is interesting to ask what features are recognized by synthetases (the enzymes that attach methionine to tRNA^{Met}) and by the bacterial transformylase. The pattern of recognition by synthetases is curious: the enzyme from either *E. coli* or the cytoplasm of eucaryotic cells recognizes both tRNA$_i$^{Met} and tRNA$_m$^{Met} from the homologous source, but recognizes only tRNA$_i$^{Met} from the heterologous source (336). Extensive chemical modification studies with *E. coli* Met-tRNA$_i$^{Met} have identified only four positions that are crucial for recognition by the cognate synthetase: nucleotides G$_{70}$ and C$_{75}$ in the acceptor stem and nucleotides C$_{34}$ and A$_{35}$ in the anticodon loop (reviewed in reference 401). The pattern of recognition by the transformylase from *E. coli* is also distinctive: the enzyme recognizes nearly all Met-tRNA$_i$^{Met} species that have been studied (Table 4) and recognizes none of the Met-tRNA$_m$^{Met} species (336). One of the few initiator tRNAs that does not function as a substrate with *E. coli* transformylase is wheat germ cytoplas-

mic Met-tRNA$_i^{Met}$. Sequence analysis reveals that the base pair G$_2$:C$_{71}$ present in all other initiator tRNAs is replaced by U$_2$:A$_{71}$ in wheat germ cytoplasmic Met-tRNA$_i^{Met}$ (reference 133 and Fig. 2), strongly suggesting that G$_2$:C$_{71}$ forms part of the recognition site for transformylase. It is surprising that the features required for formylation have been conserved in most eucaryotic initiator tRNAs despite the absence of formylation in the cytoplasm of eucaryotic cells. A possible explanation is that transformylase recognizes (some of) the same features as synthetase.

The aminoacyl moiety (be it methionine or formylmethionine) carried by the initiator tRNA is usually detectable only on nascent polypeptides; it generally is not retained at the NH$_2$ terminus of mature proteins. In bacteria, for example, the formate moiety is quantitatively removed, and many proteins also lose the terminal methionine residue. In eucaryotes, the extent of removal of the initiating methionine in vivo varies depending on which amino acids occur in the penultimate and subpenultimate positions (427). When translation is carried out in vitro with extracts from eucaryotic cells, the NH$_2$-terminal methionine is sometimes removed from the growing polypeptide (190, 201, 272, 282, 345) and sometimes not (36, 344, 384, 428). The vast majority of eucaryotic proteins are blocked by post-translational acetylation of the (original or derived) terminal α-NH$_2$ group (36, 54) or by some alternative blocking group (532). Proteins synthesized in mitochondria are the only ones that seem to retain the original NH$_2$-terminal formylmethionine (56).

THE STRUCTURE OF MESSENGER RNA (mRNA)

mRNA Structure in Procaryotes

Polycistronic mRNAs are the rule. Bacterial and bacteriophage genes are nearly always transcribed in clusters, and the resulting polycistronic primary transcript can function as mRNA. Procaryotic mRNAs usually have a 5' untranslated sequence, ranging in length from 26 to over 200 nucleotides, preceding the first coding sequence. Only two exceptions have been found so far in which the initiator codon occurs directly at (368) or a few nucleotides in from (361) the 5' end. A short 3' untranslated segment follows the last coding sequence in bacterial transcripts. Some procaryotic mRNAs terminate with oligo- or poly(A) (148, 149, 322), although that feature is far less common in procaryotes than in eucaryotes. In most bacterial messengers, an intercistronic region, ranging in length from 1 to 400 nucleotides, separates one coding sequence

from the next; but there are numerous examples of coding sequences that directly abut one another or even overlap. Although the length of the nontranslated segments flanking procaryotic genes is quite variable, the overall trend is for such sequences to be short. In the genome of bacteriophage φX174, for example, 5,169 nucleotides out of 5,386 encode amino acids (397), and in the leftmost one-third of bacteriophage T7 DNA, the translated nucleotides total 10,500 out of 11,200 (98). It is not uncommon to find a one-base-pair overlap between the terminator codon of one cistron and the initiator codon of the next, e.g. UGAUG. This is such a neat design that one is tempted to ascribe to it some important regulatory significance. In cases where the terminator codon of one cistron overlaps the initiator codon of the next, there is no indication that translation of the upstream cistron interferes with translation of the one that follows (96, 410). There are examples in which translation of the downstream member depends on prior translation of the preceding region (328), but this dependence is not always found with overlapping cistrons and is sometimes found with nonoverlapping (but adjacent) cistrons (347). In short, it does not rationalize the overlapping arrangement of terminator and initiator codons. It might be thought that the overlapping pattern would be highly conserved if it had regulatory significance, but that argument is inconclusive, since the structure of intercistronic regions is conserved in some cases (e.g., between the *trp* operon of *E. coli* and that of *Salmonella* [73]) but not in others (143). Müller and Wells (316) have experimentally expanded and contracted the intercistronic region between φX174 genes *J* and *F* with minimal effects on phage growth. Thus, a short (or even a long) untranslated sequence between genes may not be disadvantageous from the point of view of translation. Contraction of the untranslated region between cistrons most likely reflects an evolutionary drive toward genetic economy.

Although the tendency for procaryotic genes to directly abut or overlap one another has no clear regulatory implications, the polycistronic structure of procaryotic mRNAs has profound regulatory consequences for both transcription and translation. The phenomenon whereby a block in the translation of one gene results in failure to transcribe downstream genes is widespread, and it has been described elsewhere (2, 538). A less common phenomenon occurs with some polycistronic mRNAs in which the ability to translate one cistron is dependent on prior translation of the preceding cistron. In the case of coliphage MS2 RNA, expression of the 3'-proximal polymerase cistron requires prior

translation of the preceding coat protein cistron. The explanation is that the polymerase ribosome binding site is complementary to a stretch of 21 nucleotides that lie within the coat protein cistron (308). As a ribosome traverses the coat cistron, that base-paired region is disrupted, thereby exposing the polymerase initiation site. The mechanism underlying other examples of translational coupling is less well understood. The phenomenon occurs in the *trp* and *gal* operons (328, 410) and in each of the operons encoding *E. coli* ribosomal proteins (335). Here again the explanation might be that translation of an upstream cistron alters the conformation of the polycistronic messenger, thereby exposing the initiation site of the next cistron. An alternative view is that ribosomes cannot initiate de novo at downstream cistrons. Instead, upon completing transit of the first cistron, the same ribosome would advance to the next reading frame and "reinitiate." Unfortunately, there is no substantive evidence to support that intriguing mechanism, and some evidence contradicting it has been described (550). An even more complicated reinitiation scheme has been invoked to explain how the lysis gene of coliphage MS2 gets expressed (224). Although the available evidence is insufficient to reveal the exact mechanism(s), it is clear that the polycistronic character of procaryotic mRNAs permits the translation of cotranscribed genes to be closely coupled.

AUG is the usual, but not the only, initiator codon. In the classic ribosome binding experiments that were carried out in the 1960s with trinucleotides as templates, AUG, GUG, and UUG were found to stabilize the binding of fMet-tRNA to *E. coli* ribosomes (74). By using defined polynucleotides as templates in a phasing assay, Thach et al. (485) identified UUG and ACG also as functional initiator codons, although their efficiency was much lower than that of AUG. Nucleotide sequence analyses of bacterial and phage mRNAs subsequently confirmed that procaryotic ribosomes are not limited to using AUG as the initiator codon. The current catalog of procaryotic ribosome binding sites includes 12 genes that initiate with GUG, three that initiate with UUG, and one that uses AUU as the initiator codon (Table 5). There are, in addition, many examples of GUG and UUG triplets that function as "restart sites" following nonsense codons. In some mutant ribosome binding sites, a low level of function persists even when the initiator codon is changed to AUA. Although the data in Table 5 reveal a surprising flexibility in the composition of initiator codons, one should not conclude that GUG, which is the most frequently encountered al-

ternative, can be substituted ad libitum for AUG. Whereas an AUG triplet in position 101 to 103 of the T4 *rIIB* gene (mutant N24*) is a functional initiator codon, for example, a GUG triplet in the same position (mutant N24) does not work (323). (These mutants also differ from each other in two positions preceding the potential initiator codon: CA<u>AUG</u>A (mutant N24*) versus UG<u>GUG</u>A (mutant N24). The difference in the flanking nucleotides might be partly [or wholly] responsible for failure to initiate at GUG in mutant N24.) The fact that GUG, UUG, and AUU are used as codons so infrequently in natural procaryotic mRNAs suggests that these alternative initiator codons function less efficiently than AUG at some step in the initiation process. Perhaps to compensate for this weakness, some messengers that use initiator codons other than AUG have lengthy "Shine-Dalgarno sequences." This is certainly not true of the mutant and restart sites listed in Table 5, but those sites nearly always function inefficiently.

Sequence context around initiator codons. The primary sequences flanking functional initiator codons in bacterial and phage mRNAs have been scrutinized to identify features that might be recognized by ribosomes. Besides the initiator codon itself, the only other (nearly) universal feature is a purine-rich sequence centered about 10 nucleotides upstream from the initiator codon. As first noted by Shine and Dalgarno (431), that purine tract is complementary to the 3'-terminal sequence of 16S rRNA; its pivotal role in initiation is discussed at length below. Although the functional significance of other semi-conserved features in procaryotic ribosome binding sites is obscure, the following features have been noted.

Apart from the purine-rich Shine-Dalgarno sequence, the remainder of the ribosome binding site is deficient in G residues (400, 465). A or U is the preferred nucleotide in every position between the Shine-Dalgarno sequence and the initiator codon, as well as immediately upstream from the Shine-Dalgarno site (400).

A computer-assisted survey of ribosome binding sites revealed that the choice of nucleotides is especially nonrandom in position −3, where A is preferred, and positions +4 to +7. (The initiator codon is designated +1 to +3, and numbering continues [+4, etc.] into the coding sequence. The nucleotide immediately preceding the initiator codon is designated −1.) The preferred sequence in positions +4 to +7 is either GCUA or AAAA (465). As noted by Stormo et al. (465), the tRNAs that read the codons GCU and AAA are among the most abundant in *E. coli* cells; this may rationalize the observed sequence preference at the beginning

TABLE 5. Initiation at codons other than AUG in bacterial and phage mRNAs

Initiator codon	Natural initiation site[a]	Initiation site created by point mutations or activated by a preceding nonsense mutation
GUG	AGGAGGUuugAccuGUGc (Coliphage MS2 A-protein [115])	GUuucccgcGUGg (Internal initiation site 23 in E. coli lac repressor gene [448])
	GGAGAuuuucAAcGUGA (Coliphage M13 gene III [525])	AACuGuAUUcAAGUGg (Internal initiation sites III and VI in rIIB cistron of coliphage T4 [323])[b]
	GGAGAcUCUaAGUGg (Coliphage T7 protein 2.8 [98])	AGGA/AuUGGCAAGGUGu
	AGGAGUgcAuuAGUGg (E. coli ribosomal protein S13 [364])	
	GGGAGcAAAUCCGUGA (E. coli RNA polymerase,β' subunit [342])	
	GAGGCAuuguGUGUGA (E. coli atp operon, gene 4 [128])	
	GGAGAAAUAAAGUGA (E. coli alkaline phosphatase [229])	
	GGAGGAAuguCGUGc (E. coli fumarate reductase [76])	
	GGGUGGUGAAuGUGA (E. coli lactose repressor [448])	
	AGGAAuAuAgccGUGu (E. coli elongation factor Tu [542])	
	AGGAuggGUGUGA (E. coli resistance transfer factor repAI gene [47])	
	GAGAAuAAAcGUGg (E. coli histidyl-tRNA synthetase [105])	
UUG	GGGGAGUuGGACCUUGg (E. coli ribosomal protein S20 [289])	AAGGuGuAuccGUUGA (Internal initiation site VII in rIIB cistron of phage T4 [323])[b]
	UAAGGGGGUcAcGUUGA (E. coli reduced nicotinamide adenine dinucleotide dehydrogenase [544])	GGGCAAAcAGUCGUUGc (Internal initiation site 62 in E. coli lac repressor gene [448])
	GGAGGGuuuAuuUUGA (S. aureus β-lactamase [300])	
AUU	GGAGGAAUAAGGuAUUA (E. coli initiation factor IF-3 [390])	
AUA		UAAGGAAAAuuAUAu (Phage T4 rIIB mutant HD263 [23])[c]
		GGAAuuuuAUAA (S. typhimurium his leader peptide, mutant 9856 [210])[d]
		GGGUcAAuuuGAUcAUAA (Coliphage Qβ coat protein, mutant C3/C4 [483])[e]

[a] The initiator codon and presumptive Shine-Dalgarno sequence are shown in large type.

[b] Although genetic evidence suggests that the indicated GUG and UUG condons in the T4 rIIB gene are functional initiation sites, the assignments have not yet been confirmed by direct sequencing of the restart proteins.

[c] At 25°C ribosomes initiate inefficiently at the mutant ribosome binding site in HD263; at 37°C the site is nonfunctional.

[d] Translation of mutant 9856 is far less efficient than that of the wild type, in which the initiator codon is AUG.

[e] Mutant C3/C4 sequence, which is functional, differs from the wild-type sequence in two positions: AUGG in the wild-type phage was changed to AUAA in mutant C3/C4. Another mutant RNA that had the sequence AUAG failed to bind to ribosomes in vitro.

of the protein coding region. There are a few other positions in procaryotic ribosome binding sites in which the choice of nucleotides differs somewhat from random, but the deviations are less striking than those just described.

Several years ago, Atkins (12) noted that a terminator codon—either UAA or UGA, but never UAG—frequently occurred within the span of 15 nucleotides preceding the initiator codon. As the catalog of ribosome binding sites has grown, however, exceptions to the Atkins' rule have accumulated (144). Although his notion that terminator signals are involved in translation initiation may yet be vindicated, it seems more likely that the nonrandom nucleotide composition of initiation sites is responsible for the preponderance of UAA and UGA triplets.

Some ribosome binding sites have a hairpin-like structure upstream from the initiator codon, which has been postulated to serve as a recognition element for ribosomes (16, 414). Stable hairpins are not a regular feature of all procaryotic initiation sites, however. Although the sequence of the trpC-trpB junction in Salmonella typhimurium can form a stem-and-loop structure (414), the required symmetry has not been conserved in the corresponding region of the E. coli trp operon (73). Deletion of the hairpin structure preceding the rIIB gene of coliphage T4 was found to have little or no effect on expression of that gene (433). The available evidence does not support the hypothesis that stem-and-loop structures serve as positive recognition elements for binding of ribosomes.

mRNA Structure in Eucaryotes

Monocistronic mRNAs are the rule. mRNAs in eucaryotic cells are invariably monocistronic, and the size of the mRNA is usually proportional to the size of the encoded protein. This is true also of most plant and animal viral mRNAs, although there are, among viral messengers, some exceptions to the monocistronic rule. For example, quite a number of viral mRNAs have the form diagramed in Fig. 3b. These mRNAs are structurally polycistronic: they encode two or more nonoverlapping proteins. But in all such cases, save one, translation is limited to the 5'-proximal cistron. (The only documented exception is simian virus 40 late 16S mRNA, in which the 62-amino acid "agnoprotein" is encoded upstream from capsid protein VP1 and both proteins are translated [207].) These mRNAs therefore conform to the rule for eucaryotes in that they are functionally monocistronic. A small number of eucaryotic mRNAs constitute a third category, diagramed in Fig. 3c. These mRNAs encode and express two proteins. In most cases, this results from initiation at the first and second AUG triplets—in contrast with the majority of eucaryotic mRNAs, in which only
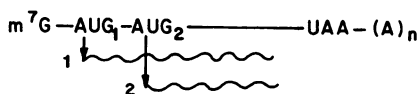

(a) Structurally and functionally monocistronic

m^7G — AUG ———————— UAA — $(A)_n$

All characterized cellular mRNAs
Many animal virus mRNAs, including those of reovirus, influenza virus, vesicular stomatitis virus, and vaccinia virus (1972)
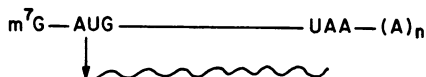
(b) Structurally polycistronic functionally monocistronic

m^7G — AUG_1 ——— UAA — AUG_2 — UAA — $(A)_n$

Semliki Forest virus 42S genome (140)
Rous sarcoma virus genome (523)
Polyoma virus late 19S mRNA (435)
Brome mosaic virus RNA-3 (429)
Tobacco mosaic virus genome (195)
Many of the adenovirus late mRNAs (9)
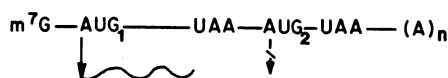
(c) Functionally dicistronic

m^7G — AUG_1 – AUG_2 ———— UAA – $(A)_n$

Simian virus 40 late 19S mRNA (358)
Herpes simplex virus thymidine kinase mRNA (366)
Adenovirus E1b mRNA (42)
Bunyavirus s-RNA (34)
Reovirus s1 mRNA (244)

FIG. 3. Schematic representation of the structure and expression of eucaryotic mRNAs. mRNA is represented by a straight line, and the encoded protein is represented by a wavy line. Three categories of mRNAs are illustrated on the left, and examples of each are listed on the right. Messengers in category a have a single long open reading frame. Those in category b have two open reading frames, indicated by AUG_1 and AUG_2, but only the protein encoded nearest the 5' end is produced. Messengers in category c encode and direct synthesis of two proteins. The second protein encoded by mRNAs in category c is sometimes merely a shorter version of the first, as in simian virus 40 VP2 and VP3. In other cases, however, the two functional initiator codons lie in different reading frames and two unrelated proteins are produced (34, 42). Inclusion of reovirus s1 mRNA in category c is tentative. It is known that ribosomes in vitro protect two distinct AUG-containing sites in s1 mRNA, but the expected two proteins have not yet been sought or found.

the 5'-proximal AUG functions as an initiator codon. Below I will suggest an explanation for these unusual bifunctional mRNAs. In view of their rarity, they pose only a minor contradiction to the monocistronic rule.

The monocistronic rule for gene expression in eucaryotes states not only that a single mRNA directs synthesis of a single protein but also that the protein encoded closest to the 5' end is the one that gets expressed. This holds true for all of the naturally occurring viral mRNAs listed in Fig. 3b, as well as for artificially constructed messengers that encode two proteins (444). The 5'-terminal restriction is imposed not by the structure of eucaryotic mRNAs but rather by the properties of eucaryotic ribosomes. This was cleverly demonstrated by incubating wheat germ ribosomes with a polycistronic mRNA from bacteriophage lambda (383). Whereas *E. coli* ribosomes readily translate the first and second cistrons in that message, wheat germ ribosomes translate only the first. Failure to translate downstream cistrons implies that eucaryotic ribosomes can neither bind directly to internal initiation sites nor reinitiate at a second cistron after traversing the first. Clearly, eucaryotic ribosomes operate under restrictions that do not occur in procaryotes.

The inability of eucaryotic ribosomes to initiate at sites in the interior of a message means that eucaryotic cells cannot coordinate synthesis of a set of proteins by clustering the genes behind a single transcriptional promoter. Polycistronic transcripts, which are the predominant regulatory device used by procaryotes, will not work in eucaryotes. In view of this limitation, it is interesting to ask what compensatory mechanisms eucaryotic cells have devised to coordinate gene expression. One answer (clearly not the only one) is that eucaryotic genes are sometimes fused, thus producing, in lieu of a polycistronic mRNA, a "polyprotein" which is derived from a single longsome cistron. Tryptophan synthetase is one such example: the α subunit (M_r 28,727) and β_2 subunit (M_r 42,756), which are encoded by separate genes in *E. coli*, are fused in *S. cerevisiae* to form a bifunctional protein of M_r ~76,000 (549). Other examples of fused genes encoding multifunctional proteins are the *arom* gene cluster in *Neurospora*, which includes five enzymes involved in the polyaromatic biosynthetic pathway (136), and the *his4* gene in *S. cerevisiae* which encodes a trifunctional protein (225). Perhaps the most impressive example is mammalian fatty acid synthetase (M_r 240,000), which corresponds to a set of seven individual polypeptides in *E. coli* (296)! Although the rationale behind gene fusion is more amenable to guessing than to experimentation, it is possible

that fusion of genes compensates for the inability of eucaryotic ribosomes to use polycistronic transcripts for the coordinate synthesis of polypeptides.

AUG is the only initiator codon. In contrast with procaryotes, eucaryotic ribosomes seem to initiate exclusively at AUG codons. The inability of eucaryotic ribosomes to initiate at GUG was first recognized by Stewart et al. (459), who showed that a point mutation converting the AUG initiator codon to GUG abolished translation of the iso-1-cytochrome *c* gene in *S. cerevisiae*. More extensive analyses of yeast mutants failed to identify any codon other than AUG that was capable of initiating translation of iso-1-cytochrome *c* (426). The current catalog of ribosome binding sites from more than 200 eucaryotic mRNAs (242; unpublished compilation) includes not a single example of initiation at a codon other than AUG. It is interesting, however, that several mammalian pseudogenes have been identified in which the initiator codon has been mutated to GUG (208, 367) or AUA (139). But it would be premature to conclude that the mutation in the initiator codon is responsible for failure to express those genes, since there are other sequence differences between the pseudogenes and their functional counterparts. Ribosome binding studies carried out in vitro with synthetic oligo- or polynucleotides at low magnesium concentrations have confirmed that GUG cannot substitute for AUG in the formation of initiation complexes with mouse ascites (53) or wheat germ (243) ribosomes. Curiously, it appears as if yeast Met-tRNA$_i^{Met}$ functions with either AUG or GUG as the initiator codon in a heterologous system with *E. coli* ribosomes (370). Nonetheless, when all of the components of the translational machinery are derived from eucaryotes, AUG is the only codon that functions in initiation.

Post-transcriptional modifications. Eucaryotic transcripts undergo multiple processing reactions before (or during) their transport from the nucleus into the cytoplasm. Since this subject has been reviewed frequently of late, the brief summary that follows will highlight only those features which (may) relate to translational initiation. The principal modifications involved in generating mature mRNAs in eucaryotes are addition of a methylated cap to the 5' terminus of the transcript, splicing, and addition of a poly(A) "tail" to the 3' terminus. There are exceptional mRNAs lacking each of these modifications, but the majority of eucaryotic cellular and viral messengers are capped, spliced, and polyadenylated.

The 5'-terminal cap structure consists of m^{7}G linked via a 5'-5' triphosphate bridge with the

first encoded nucleotide in the messenger (421). The cap serves two functions: it dramatically enhances binding of 40S ribosomal subunits to mRNA (19, 421), and it is a major determinant of mRNA stability (123, 315). No functional uncapped mRNAs have yet been identified in eucaryotic cells, although there are a few plant and animal viral mRNAs that function without a cap. The consequences of adding m^{7}G to a naturally uncapped viral mRNA are somewhat variable. In the case of satellite tobacco necrosis virus RNA, introduction of a cap had no detectable effect on translation in vitro (438). In a more artificial situation, in which eucaryotic ribosomes were required to translate a procaryotic messenger, however, efficient translation depended on prior capping of the mRNA (346). The consequences of removing the m^{7}G moiety from a naturally capped mRNA are more predictable. There is always a significant decrease in translational efficiency, but the magnitude of the effect depends on which messenger is tested, and in which cell-free extract (277, 531). The variable extent to which the methylated cap contributes to translational efficiency has also been shown by assaying the inhibitory effect of cap analogs on in vitro translation (522, 527). In addition to the terminal m^{7}G moiety, the penultimate and subpenultimate nucleotides (N and N' in the structure m^{7}GpppNpN' [p indicates phosphate]) are often methylated, particularly in mRNAs from higher eucaryotes (421). There is no discernible effect on mRNA function in vivo when those modifications are prevented (87, 214).

In many eucaryotic genes, the protein coding sequence is interrupted by one or more intervening sequences (introns), making it necessary to splice the primary transcript to generate in the mature mRNA an uninterrupted reading frame for translation. The mechanism of splicing and its critical role in regulating gene expression are described in several recent reviews (81, 119, 267, 420). There are only a few examples of introns occurring within the 3' noncoding region of eucaryotic genes (260, 327, 511), but there are many examples of viral (260, 520, 548) and cellular (6, 65, 114, 120, 186, 302, 320, 330, 352, 545) genes in which the 5' noncoding sequence is interrupted. Whereas failure to remove intervening sequences from the coding portion of a transcript has obvious consequences for translation, it is not clear what would result from failure to remove introns from the 5' untranslated region. The scanning model (described below) predicts that if an intron that lies in the 5' untranslated portion of a message contains an AUG triplet, that AUG codon might be selected in preference to the natural initiation site were

the intron not removed. In one case where this was tested by restructuring the DNA, retention of a portion of the small intervening sequence in rat insulin II mRNA drastically decreased, but did not abolish, initiation at the normal AUG codon that lies downstream (278). Adenovirus mRNAs provide a more natural test of the hypothesis. A small percentage of late adenovirus transcripts retain a sequence, called the i leader, which is spliced out of the 5' end in the majority of late transcripts. In the mRNA molecules that retain the i leader sequence, ribosomes initiate at an AUG codon contained within that sequence (510) rather than at the downstream AUG triplet used in the majority of late transcripts (4). Thus, the manner of splicing near the 5' end of a transcript can have drastic consequences for mRNA function.

Most, although not all, eucaryotic cytoplasmic mRNAs terminate with a poly(A) segment that is typically 50 to 150 nucleotides in length. The poly(A) sequences on yeast mRNAs tend to be somewhat shorter (357). Brawerman (46) has recently reviewed what is known about the synthesis and metabolism of the poly(A) tail. Its role in translation remains purely conjectural, since mRNAs deficient in poly(A) can still be translated (46, 443, 551). The poly(A) segment seems to enhance the stability of some mRNAs (193, 194, 551), although other messengers lacking poly(A) are nonetheless stable (443).

Untranslated sequences at the 5' end. The length of the 5' noncoding "leader" sequence in eucaryotic mRNAs ranges from 3 (226) to 742 (231) nucleotides. The extremely short and extremely long leaders are rare and are usually confined to viral messengers. Most cellular mRNAs have 5' noncoding sequences in the range of 40 to 80 nucleotides (242). Although eucaryotic ribosomes can recognize an AUG triplet that lies just three nucleotides from the 5' terminus, in such mRNAs initiation is not restricted to the 5'-proximal AUG codon. For example, initiation occurs at the first and the second AUG triplets in an immunoglobulin mRNA that has the sequence m^{7}GpppGAAAUG CAUCACACCAGCAUGG (226). Thus, the precision of initiation seems to be impaired if the sequence preceding the AUG codon is too short. (It is not clear whether eucaryotic ribosomes can recognize an AUG triplet that is separated from the m^{7}G cap by fewer than three nucleotides. The 42S genomic RNA of Semliki Forest virus, for example, has the structure m^{7}GpppAUG [264, 524], and porcine gastrin mRNA probably has only one nucleotide between the cap and the first AUG codon [543]. In both Semliki Forest virus and gastrin mRNA, the cap-proximal AUG triplet is followed closely

by an in-frame terminator codon; the long open reading frame in those mRNAs begins with another AUG located not far downstream. It is clear, therefore, that some ribosomes initiate at an AUG triplet downstream from the cap-proximal AUG in those mRNAs, but it has not been proven that no ribosomes initiate at the cap-adjacent AUG triplet. The small peptide that would result would be very difficult to detect.) What about the opposite extreme? There is no evidence that translation is augmented by the presence of an unusually long 5' noncoding sequence, although that idea has its advocates (262). In contrast, there are hints that the long 5' noncoding sequence in some mRNAs may have a deleterious effect. I do not think that the adverse effect is due to length per se, since two forms of adenovirus fiber mRNA are equally translatable in vitro, even though one has an extra 180 nucleotides in the leader region (94). Moreover, the Drosophila heat shock mRNAs are among the few cellular messengers with very long 5' untranslated sequences—111 to 253 nucleotides (186, 198, 199)—and heat shock mRNAs are translated at least as efficiently as other cellular mRNAs (183, 271). Although the length of the 5' noncoding sequence per se may not adversely affect translation, a long leader sequence might be deleterious if it contains excessive secondary structure or if it contains AUG triplets which might deflect ribosomes from the authentic initiation site that lies farther downstream. In the long leader sequences of poliovirus (231), Rous sarcoma virus (475), Semliki Forest virus (264) and simian virus 40 (260), for example, several AUG triplets occur upstream from the authentic initiation site. According to the hypothesis just advanced, those messengers should be translated inefficiently (which is true, at least, for poliovirus and Semliki Forest virus), and aberrant transcripts that lack portions of the normal 5' noncoding sequence should be translated more efficiently than the "standard" form of mRNA. The latter point has been demonstrated with simian virus 40 late mRNAs in vitro (67).

The 5' untranslated regions of eucaryotic mRNAs show enormous variation in sequence as well as length. Even between related pairs of genes, in which the coding sequences are closely conserved, 5' noncoding sequences are sometimes highly divergent (71, 72, 222, 298, 313). Moreover, the translatability of a given mRNA does not seem to be perturbed when the 5' noncoding region is altered by deletion, insertion, or recombination (reviewed in references 242 and 245). Although the overall heterogeneity of 5' noncoding sequences is remarkable, the following five conserved (or semiconserved) features have been identified.

(i) A striking feature of eucaryotic mRNAs is that there are usually no other AUG triplets preceding the functional initiator codon. Two years ago, when the sequences of 130 eucaryotic mRNAs were compiled and published (245), I could find only 15 "exceptional" messengers, in which ribosomes did not initiate at the AUG codon closest to the 5' terminus. A more recent compilation of 200 sequences (M. Kozak, unpublished) uncovered only three additional mRNAs in which AUG triplets occur upstream from the functional initiation site (75, 177, 543). (This list does not include human serum albumin mRNA, although the complementary DNA sequence reported for that gene by Lawn et al. [259] contains two ATG triplets upstream from the functional initiator codon. The ATG triplets lie in a region that was probably scrambled during synthesis of the complementary DNA; it is unlikely that they are actually present in the mRNA [93; R. M. Lawn, personal communication]. The sequence reported for pre-α-lactalbumin complementary DNA also has two ATG triplets within the 5' untranslated region [168]. I have temporarily excluded that gene from the tabulation until the error-prone 5' portion of the complementary DNA sequence has been confirmed.) Thus, the first-AUG rule holds for ~90% of eucaryotic mRNAs, including many with very long 5' leaders (84, 124, 198, 199, 330, 386, 423, 520, 548). A similar bias is not seen in bacterial mRNAs.

(ii) Functional initiator codons in eucaryotic mRNAs occur in a restricted sequence context. A preliminary survey of sequences flanking AUG initiator codons revealed two conserved positions: a purine (usually A) frequently occurs three residues before the AUG codon, and a purine (usually G) often follows the AUG (243). Table 6 shows the distribution of initiator and non-initiator AUG triplets with respect to the nucleotides in positions −3 and +4. Most functional initiator codons fall among the top five sequences listed in Table 6; i.e., the preferred sequence context for initiation is either ANNAUGN or GNNAUGR (R indicates a purine). On the other hand, nonfunctional AUG triplets that occur in the 5' noncoding region of the exceptional mRNAs mentioned above cluster among the bottom four sequences listed in Table 6. In other words, although AUG triplets do occur upstream from the functional initiation site in a small number of eucaryotic mRNAs, the sequence context around the nonfunctional upstream AUG triplets differs from that flanking functional initiator codons. As a control, I included in the survey AUG triplets that code for methionine at internal positions in polypeptide chains. Internal AUG codons distribute more or less randomly with respect to flanking nucleotides (Table 6).

(iii) The sequence CACACA occurs about 10 nucleotides upstream from the AUG initiator codon in a variety of yeast mRNAs (88, 549). Based on analysis of certain iso-1-cytochrome *c* mutants in *S. cerevisiae*, Stiles et al. (462) postulated that the CACACA motif may form part of the recognition sequence for yeast ribosomes; but many other yeast genes lack the indicated sequence (5, 28, 72, 88, 124, 313, 388, 468, 516), and genes from higher eucaryotes, which lack the CACACA sequence, are efficiently translated when properly introduced into *S. cerevisiae* (498). The significance of that sequence is doubtful.

(iv) In mRNAs from lower eucaryotes, such as yeasts and slime molds, the 5' untranslated region is characteristically AU rich (28, 72, 85, 124, 185, 298, 313, 362). The AU content of some leader sequences exceeds 90%. In mRNAs from higher eucaryotes, the composition of the 5' noncoding region is usually less biased, except for a notable deficiency of G residues (243). The significance of the G-poor region preceding the initiator codon is unknown. It is curiously reminiscent of procaryotic ribosome binding sites.

(v) There are hints of conserved sequence(s) adjacent to the m^{7}G cap, at least in some messengers. The penultimate nucleotide (N in the sequence m^{7}GpppN) is nearly always a purine, most often A (18, 48, 424). Exceptional mRNAs, however, initiate with a pyrimidine (124, 218, 319). More extensive homology at or near the 5' terminus is often seen within a set of related mRNAs. For example, all five histone mRNAs in *Strongylocentrotus purpuratus* begin with the pentanucleotide AUUCR (474), after which the sequences diverge. The sequence CUUYUG occurs seven nucleotides downstream from the cap in all mammalian β-like globin genes (104). A common sequence (RUCAUU) is found at the 5' terminus of all members of the chorion gene family in silk moths (211), and a very similar sequence (AUCAGU) initiates each mRNA species encoding the cuticle proteins in *Drosophila* (439). Recent experiments suggest that the conserved sequence at the cap site in some mRNAs comprises part of the promoter for transcription (480). An additional role in translation is not ruled out, however.

Untranslated sequences at the 3' end. The heteropolymeric 3' noncoding region [i.e., the sequence between the translation termination site and the poly(A) addition site] typically ranges from 50 to 150 nucleotides, although its length exceeds 1,000 nucleotides in some mRNAs (219). Comparison between related genes reveals that 3' noncoding sequences are less conserved than coding sequences (104, 203, 284, 285, 306, 313, 362, 419), with a few notable

TABLE 6. Sequence context around AUG triplets in eucaryotic mRNAs[a]

Sequence[b]	No. of mRNAs containing sequence as:		
	Functional initiator codon	"Nonfunctional" upstream AUG	Internal AUG codon
ANNAUGG	74	1[c]	25
ANNAUGA	38	0	26
ANNAUGY	29	0	27
GNNAUGG	18	2[d]	39
GNNAUGA	4	0	18
GNNAUGY	4	4	38
YNNAUGG	11	4	25
YNNAUGA	1(?)	7	23
YNNAUGY	1	14	47

[a] The mRNAs that were included in the survey of functional initiator codons are identified in references 243 and 245. The nonfunctional upstream AUGs are from 17 mRNAs, described in the text, in which one or more AUG triplets precedes the start of the protein coding sequence. (The first, presumably nonfunctional, AUG triplet in porcine gastrin mRNA is not included in this tabulation because it is separated from the cap by only one nucleotide [543].) The internal AUG codons are from 9 viral and 15 cellular mRNAs, which are identified in reference 245.

[b] Y indicates a pyrimidine.

[c] An AUG codon flanked by the indicated sequence lies upstream from the functional initiator codon in the *src* mRNA of Rous sarcoma virus (475).

[d] The indicated sequence occurs twice upstream from the initiator codon for VP1 in the late 16S mRNA of simian virus 40 (260).

exceptions (178). Even more surprising than the variation in 3' noncoding sequences among different genes is the observation that the mRNA population encoding a single protein sometimes displays marked 3'-terminal heterogeneity. For example, mRNAs encoding dihydrofolate reductase can be fractionated into at least four size classes, in which the length of the 3' untranslated region varies from 80 to about 930 nucleotides (416). Although all forms of dihydrofolate reductase mRNA extracted from mouse cells can be translated in vitro, it is not known whether all are translated with equal efficiency. Similarly, although in vitro studies revealed that mRNA from which the 3' noncoding region had been enzymatically removed could still be translated (249, 443), the efficiency of translation of the truncated messenger was not evaluated. In short, the idea that 3' noncoding sequences serve a useful, albeit dispensable, function in translation is still tenable. Indeed, although sequence conservation within the 3' untranslated region is nearly always lower than in the coding region, the mutation rate within some portions of the 3' noncoding segment is lower than that of nonfunctional DNA (294, 309).

Structure of Organellar mRNAs

The following description pertains to poly-some-associated transcripts which are believed to function as mRNA in mitochondria. Since the putative mRNAs have not yet been purified and translated in vitro, however, their functionality remains to be proven. Putative mRNAs from HeLa cell mitochondria are uncapped (160). They carry a 3'-terminal poly(A) segment which is not encoded in the DNA template. (Since both mRNAs and rRNAs are oligoadenylated in mito-chondria [14, 91], adenylation may be an integral part of the transcription termination mechanism rather than a requirement for mRNA function.) It is remarkable that the DNA sequences of many mammalian mitochondrial genes do not contain complete translational termination signals. Instead, a UAA terminator codon is created upon addition of the poly(A) tail (10, 11). Mammalian mitochondrial mRNAs have two unusual features that bear on the mechanism of translational initiation: (i) the initiator codon is frequently AUA or AUU, instead of the canonical AUG (33, 314), and (ii) the AUN initiator codon is either directly at or very close to the 5' end of the transcript (14, 314). Most of the mRNAs in mammalian mitochondria are mono-cistronic, although there seem to þe a few exceptions (11). The gene-size mRNAs appear to be derived by cleavage from a single polycistronic precursor (14).

The structure of yeast mitochondrial mRNAs differs in several important respects from that just described for mammalian mitochondria. The initiator codon is AUG in all yeast mitochondrial genes that have been sequenced. (*Aspergillus* mitochondria follow the same rule, except for the cytochrome oxidase subunit 3 gene, which initiates with GUG [326].) The protein coding sequence is preceded by an AU-rich untranslated segment, reminiscent of mRNAs from lower eucaryotes, that ranges in length from 54 to 940 nucleotides (78, 269). It is curious that some mRNAs from yeast mitochondria have the longest known 5' noncoding sequences, whereas mRNAs from mammalian mitochondria have the shortest! It is not uncommon to find nonfunctional AUG triplets within the long 5' untranslated region in mitochondrial mRNAs from yeasts and fungi (55, 287). The 3'-terminal oligo(A) segment is only about eight nucleotides long (546), and it is not clear whether that sequence is transcribed or added post-transcriptionally. The coding sequences of some yeast mitochondrial genes are interrupted by introns which are removed via an extraordinary splicing mechanism (268).

Little is yet known about the structure of mRNAs from chloroplasts. They lack a 3'-termi-nal poly(A) tail (391) and probably lack a 5'-terminal cap. At least one chloroplast messenger contains an intervening sequence (460). Although this may prove to be a rarity, the occurrence of mRNA splicing in a system which is otherwise so like that of procaryotes is remarkable. The few chloroplast mRNAs studied to date have lengthy noncoding sequences at both the 5' and the 3' ends. From the point of view of translational initiation, the most exciting finding (again based on very few examples) is that the AUG initiator codon is preceded by a purine-rich sequence that is complementary to the 3'-terminal sequence of 16S rRNA (247, 297, 554). This raises the possibility that the mechanism of initiation proposed by Shine and Dalgarno for bacteria might also extend to chloroplasts (see below).

Consequences of Perturbing mRNA Structure

Perturbation of mRNA structure sometimes alters its interaction with ribosomes. Table 7 describes various manipulations to which mRNAs have been subjected and briefly summarizes the consequences. The effects listed in Table 7 are rationalized in the following discussion of the initiation mechanism. For now, the main conclusions are that (some) perturbations of mRNA structure affect ribosome binding and that the response of eucaryotic ribosomes is quite different from that of procaryotic ribosomes.

THE INITIATION PROCESS

Brief Overview

Because the step-by-step assembly of initiation complexes has not yet been studied in organelles, the following description is confined to procaryotic and eucaryotic translational systems. The differences between procaryotes and eucaryotes in tRNA$_i$^{Met} structure and initiation factor requirements have already been discussed. Cofactor requirements also differ between the two systems. GTP is needed for Met-tRNA$_i$^{Met} binding to both procaryotic and eucaryotic ribosomes (180). When GTP is replaced by the nonhydrolyzable analog GDPCP, the consequences are similar, but not identical, in the two systems. GDPCP is able to mediate binding of Met-tRNA$_i$^{Met} and IF-2 (or eIF-2) to the small ribosomal subunit, but in the absence of GTP hydrolysis (e)IF-2 remains bound to the small subunit (290). In rabbit reticulocyte ly-sates, eIF-3 also stays attached to the 40S ribosomal subunit when GTP hydrolysis is prevented (355, 356). Retention of eucaryotic initiation factors on the small ribosomal subunit in the presence of GDPCP precludes joining of the

TABLE 7. Effects of altered mRNA structure on ribosome binding

Perturbation of mRNA structure	Effect on binding of ribosomes of:	
	Procaryotes (E. coli)	Eucaryotes
Circularization	No effect (49)	Binding abolished (236, 238)
Denaturation	Ribosomes initiate at spurious sites (276, 547) in addition to the authentic sites	No (direct) binding of ribosomes to internal sites in mRNA (77, 239, 547)
Limited cleavage	Activation of only a few sites (which are normally sequestered by conformation) (450)	Activation of many spurious initiation sites (reviewed in reference 242)
Extensive cleavage (limit digestion with T_1 or pancreatic ribonuclease)	A few oligonucleotides which are complementary to the 3′ end of 16S rRNA bind quantitatively to ribosomes (449, 455)	No selective binding of oligonucleotides complementary to 18S rRNA (408, 449, 455), although other large oligonucleotides sometimes bind (408)
Introduction of nonsense triplets, causing premature termination of the encoded protein	Reinitiation sites in the vicinity of the nonsense codon are activated (291, 323, 448)	No activation of potential initiation sites downstream from the nonsense codon (F. Sherman and J.W. Stewart, in Molecular Biology of the Yeast Saccharomyces, in press)

large ribosomal subunit (491). This differs from the case of bacteria, where the large subunit can join (290), although the resulting 70S ribosome is inactive in peptide bond formation. ATP is a required cofactor for initiation only in eucaryotic systems (27, 292, 491). ATP hydrolysis appears to be needed for binding of a 40S ribosomal subunit to the 5′ end of mRNA, as well as for the subsequent migration of 40S subunits from the 5′ "entry site" down to the AUG codon (240; see below). The ATP requirement for binding, but not for migration, is obviated by prior denaturation of the mRNA. ATP also promotes interaction of mRNA with one or more cap-binding proteins, as discussed above.

Eucaryotic initiation components assemble in a fixed order: Met-tRNA$_i^{Met}$ binds to the 40S subunit first, followed by mRNA (196, 491). This differs from the probable assembly order in procaryotes. In the absence of fMet-tRNA$_i^{Met}$, coliphage MS2 mRNA can form a stable complex with 30S ribosomal subunits from E. coli (507). However, other investigators claim that stable binding of mRNA to E. coli 30S subunits requires prior binding of fMet-tRNA$_i^{Met}$ (205). A kinetic analysis indicated that either order of binding is possible, at least in vitro (161). It is difficult to reconcile the conflicting data. Van der Hofstad et al. (501) reported the helpful observation that binding of IF-2 and IF-3 to 30S ribosomal subunits is mutually exclusive. Since

IF-2, once bound, remains ribosome associated until the 50S subunit joins, IF-3 must bind and undergo release before IF-2 attaches. This implies that mRNA binding, which is mediated by IF-3, necessarily precedes IF-2-mediated fMet-tRNA$_i^{Met}$ binding in bacterial systems.

In cell-free extracts containing an elongation inhibitor, such as sparsomycin, ribosomes bind very tightly to mRNA, protecting from nuclease attack a small portion of the messenger that usually represents the initiation site for protein synthesis. The size of the ribosome-protected fragment ($\sim$30 nucleotides) and the position of the initiator codon (approximately in the center of the protected fragment) are similar with either 70S procaryotic or 80S eucaryotic ribosomes. There are unusual circumstances under which ribosomes protect sequences that are not the sites where peptide bond formation initiates. The nature of those spurious sites is very different, depending on whether procaryotic or eucaryotic ribosomes are involved. When procaryotic ribosomes are incubated with mRNA in the absence of fMet-tRNA$_i^{Met}$ (484) or when certain heterologous combinations of mRNA and ribosomes are used (452), the ribosomes select and protect purine-rich sequences that lack an initiator codon. Eucaryotic ribosomes, in contrast, tend occasionally to protect sites that lie upstream from the functional initiator codon in mRNAs that have a long 5′ untranslat-

ed region (3, 80, 117, 360). In both systems, it is possible that protection of non-initiator sites is purely artifactual and hence reveals nothing about the mechanism of protein synthesis. A less pessimistic view is that trapping of ribosomes at non-initiator sites reveals intermediate steps in the initiation pathway. According to this view, the differences between procaryotic and eucaryotic ribosomes in their selection of spurious bindings sites portend different initiation mechanisms in the two systems.

The predominant role of the small ribosomal subunit in selecting initiation sites was first shown by Lodish. Taking advantage of the fact that *E. coli* ribosomes initiate at three sites in R17 RNA (A-protein, coat protein, and polymerase), whereas *B. stearothermophilus* ribosomes recognize only the beginning of the *A* cistron (274, 450), he showed that chimeric ribosomes consisting of the small subunit from *E. coli* and the large subunit from *B. stearothermophilus* (in the presence of initiation factors from either organism) initiate at all three sites in R17 RNA (275). (A logical extension of this approach was to dissociate and then reconstitute the small ribosomal subunit to determine which of its components are involved in mRNA recognition. The answer is both proteins and, to a lesser extent, 16S rRNA [145, 175]. The details of how some ribosomal proteins mediate mRNA binding are just beginning to emerge [15, 200a, 505, 545a]. Much more is known about the role of 16S rRNA, as described below.) There is additional evidence that, in both procaryotes and eucaryotes, selection of initiation sites is accomplished primarily by the small ribosomal subunit. When large ribosomal subunits are omitted from a reaction or prevented from joining, the small subunit alone selects and protects the same sites in mRNA as are protected by the corresponding 70S or 80S ribosome. (Whereas the small ribosomal subunit from bacteria protects an mRNA fragment which is identical to that protected by 70S ribosomes (263), the small subunit of eucaryotic ribosomes protects a significantly bigger mRNA fragment than that protected by 80S ribosomes [246]. The difference in size is probably due to the large number of initiation factors associated with the eucaryotic 40S subunit; the factors are released when the 60S subunit joins. The main point to note here is that the larger sequence protected by 40S ribosomal subunits always includes the sequence protected by 80S ribosomes.) Whereas the small ribosomal subunit binds to the mRNA first and chooses the initiation site, addition of the large subunit makes the process irreversible. In some cases, joining of the large subunit seems to contribute to the fidelity of initiation by "freezing" the

small subunit at a given point in its search for the initiation site (239).

Attempts to Translate mRNAs in Heterologous Systems Suggest Dissimilarities in the Initiation Mechanisms

The limited ability of procaryotic mRNAs to be translated by eucaryotic ribosomes, and vice versa, suggests dissimilarities in the two systems. A few such experiments have also been attempted with organellar mRNAs. Negative results, which are the most frequent outcome of mixed translation experiments, must be interpreted with caution. Even if the experimental gene has been provided with appropriate signals for transcription, its expression in a foreign environment might be limited by instability of either the protein (189) or its mRNA (173). At the level of translation, expression of foreign mRNAs might be impaired if they lack an appropriate ribosome binding site or if their codon usage differs from that of the host (29, 197). Interpretation of positive results can also be less than straightforward. Not uncommonly, ribosomes initiate at many spurious sites in addition to the correct one during mixed translation experiments (227, 263). Another pervasive problem is that mRNA molecules often undergo cleavage during incubation in cell-free extracts, in which case one must try to decide whether it is the intact or cleaved form of mRNA that is the active template. An analogous problem can complicate the interpretation of in vivo experiments, since the cellular transcriptional machinery often generates multiple forms of mRNA from a given gene. In the highly artificial situation where a procaryotic gene is introduced into a eucaryotic cell, or vice versa, there is no reason to expect the most abundant mRNA species to be the functional messenger. With these caveats in mind, what can we learn from heterologous translation experiments about the mechanism of initiation by procaryotic, eucaryotic, and organellar ribosomes?

When eucaryotic mRNAs are incubated in cell-free extracts from *E. coli*, bacterial ribosomes often initiate at spurious sites in the unnatural template (331, 374, 470). There are only three well-documented cases of correct (or nearly correct) translation of eucaryotic mRNAs in cell-free extracts from *E. coli*; all involve the coat proteins encoded by plant viruses (64, 141, 232). In none of those instances was it shown, however, that *E. coli* ribosomes bind exclusively at the authentic initiation site. Although I shall argue below that procaryotic and eucaryotic ribosomes initiate via very dissimilar mechanisms, both systems nevertheless require that the target site in the mRNA be relatively free of

secondary structure. It may be this common feature that permits some degree of "recognition" of the correct initiation site by heterologous ribosomes. It also happens, just by chance, that a sequence similar to the Shine-Dalgarno sequence in procaryotes occurs upstream from the AUG initiator codon in a few eucaryotic genes. Cloned eucaryotic genes that meet this requirement are expressed quite efficiently when they are introduced into *E. coli* (5, 107, 230, 468, 469). Although the fortuitous occurrence of a Shine-Dalgarno sequence allows facile expression of those few genes, the generalization remains that most cloned eucaryotic genes in their native form fail to be expressed in *E. coli* (301, 303, 371). Efforts to reshape eucaryotic mRNAs to meet the requirements of the procaryotic translational machinery have considerably extended our understanding of what *E. coli* ribosomes look for in determining where to bind.

Many procaryotic mRNAs have been translated in extracts from eucaryotic cells (reviewed in reference 237). The efficiency of expression is usually low, however (82, 495). I shall argue below that the ability of eucaryotic ribosomes to use a potential initiation site depends on the position of that site relative to the 5' end of the messenger. The results of some mixed translation experiments support that view; for example, addition of a methylated cap converted a polycistronic mRNA from phage lambda into an efficient template for wheat germ ribosomes in vitro, but translation was limited to the 5'-proximal cistron in the phage mRNA (383). Some in vivo experiments in which cloned bacterial genes were introduced into the cytoplasm of eucaryotic cells also underscore the importance of position. When recombinant plasmids were constructed so that the first AUG triplet in the resulting transcript was the initiator codon of the bacterial protein coding sequence, the bacterial protein was translated very efficiently in the eucaryotic host (337, 409). But more complicated gene arrangements sometimes functioned, too (317). The problems in interpretation outlined above might be recalled here.

In contrast with the limited translation of most eucaryotic mRNAs by bacterial ribosomes, the ribulose bisphosphate carboxylase gene from chloroplasts is translated with phenomenal efficiency by *E. coli* ribosomes, both in vivo (126) and in vitro (43). This has been taken as evidence for features similar to those of procaryotes in the ribosome binding site of (at least one) chloroplastic mRNA.

Until recently, mitochondrial mRNAs could not be translated successfully in extracts from any source. Those negative results (reviewed in reference 40) are not suprising in view of the uniqueness of the mitochondrial genetic code. By supplementing a wheat germ extract with a tRNA fraction that compensates for the peculiar mitochondrial code, DeRonde et al. (83) recently succeeded in translating a protein related to subunit II of cytochrome *c* oxidase, encoded by yeast mitochondrial mRNA. Few conclusions can be drawn until additional experiments are carried out along these lines.

Shine-Dalgarno Interaction Between mRNA and Small-Subunit Ribosomal RNA

Role of mRNA · ribosomal RNA complementarity in eubacterial systems. In 1974, Shine and Dalgarno postulated that base pairing might occur between a purine-rich sequence just upstream from the initiator codon in mRNA and a complementary sequence near the 3' end of *E. coli* 16S rRNA (431). Steitz and Jakes (456) obtained the first direct evidence for such an interaction. They worked with initiation complexes formed in vitro between *E. coli* ribosomes and a ^{32}P-labeled fragment derived from the beginning of the A-protein cistron of coliphage R17 RNA. When they treated those mRNA · ribosome complexes with colicin E3 (a nuclease that makes a single cut 49 nucleotides from the 3' end of 16S rRNA), the 3'-terminal fragment of 16S rRNA was released as a stable complex with the ^{32}P-labeled mRNA fragment. The excitement generated by those early experiments prompted many other investigators to test the Shine-Dalgarno hypothesis further.

(i) **Summary of the evidence.** An overwhelming body of evidence now supports the role of mRNA · rRNA base pairing in the selection of initiation sites by *E. coli* ribosomes. (a) Ribosome binding sites from well over 150 bacterial and phage mRNAs have been sequenced (144, 455). Nearly all include a sequence, just upstream from the initiator codon, that is complementary to the 3' end of 16S rRNA. (The exact site in *E. coli* 16S rRNA that is most often involved in the Shine-Dalgarno interaction is the CUCC sequence shown in boldface in Table 8.) It is noteworthy that a Shine-Dalgarno sequence precedes each cistron in polycistronic mRNAs (347, 365, 539). The only natural mRNAs that unequivocally lack complementarity to 16S rRNA are the messengers encoding the C_I repressor proteins of phage lambda (368) and the closely related phage 434 (361). In both cases, the AUG initiator codon lies so close to the 5' end of the mRNA that there is no room for a Shine-Dalgarno sequence. A few other messengers, such as those encoding *E. coli* dnaG primase (434) and protein 38 of bacteriophage T4, lack a recognizable Shine-Dalgarno sequence in the usual position. In the case of T4 gene 38, a

credible (although unproven) model invokes formation of a hairpin loop to bring a distant Shine-Dalgarno sequence close to the initiator codon (144). Although most of the mRNAs sequenced to date have been from *E. coli* and its close relatives, recent analyses of mRNAs from gram-positive bacteria indicate that they, too, have extensive complementarity to the 3' end of 16S rRNA (187, 299, 300, 318), but there is not yet enough information from gram-positive organisms to assess directly the importance of the Shine-Dalgarno interaction (or the possibility of other interactions) in that system. (b) A variety of experiments indirectly implicate the 3' end of 16S rRNA in initiation. By electron microscopy, the 3' terminus of 16S rRNA has been localized to the platform region of the 30S ribosomal subunit (339, 422, 464), near the site where mRNA and initiation factors also bind (506). A sequence near the 3' end of 16S rRNA is also the target for kasugamycin (176), which is a potent inhibitor of initiation. (c) The biochemical techniques devised by Steitz and Jakes for analyzing R17 RNA were subsequently used with other messengers, permitting direct isolation of mRNA · rRNA complexes within which the purine-rich sequence preceding the initiator codon was resistant to ribonuclease attack (457). (d) The 3' end of 16S rRNA is positioned within the ribosome in a way that permits it to pair with complementary oligonucleotides. Thus, when *E. coli* ribosomes were incubated with a heterogeneous mixture of ^{32}P-labeled oligonucleotides, those oligonucleotides complementary to the 3' terminus of 16S rRNA were selectively bound (449, 455). (e) Oligonucleotides complementary to the 3' end of 16S rRNA inhibit the binding of mRNA to bacterial ribosomes (101, 482). The inhibition is specific for natural mRNAs; binding of AUG or poly(U) was not impaired. (f) Translation of procaryotic mRNAs is greatly diminished by mutations that alter or delete the Shine-Dalgarno sequence. The first such point mutations were found in coliphage T7 gene 0.3 (95). Mutations that disrupt mRNA · rRNA complementarity, thereby impairing translation, have recently been characterized in other genes (mutant 713 in reference 412; mutant zEM72a in reference 433). There is one interesting report of enhanced translation due to a point mutation that improves the potential for base pairing between mRNA and 16S rRNA (69), and there is one instance in which the Shine-Dalgarno sequence was mutagenized without apparent effect on the translatability of the mRNA (137). In the last case, although the mRNA · rRNA complementarity was reduced from eight to four contiguous nucleotides, the residual four base complementarity was in the most favorable position for interaction with 16S rRNA (see below).

(ii) Characteristics of the Shine-Dalgarno sequence in procaryotic mRNAs. Three parameters influence the efficiency of the Shine-Dalgarno interaction: the length of the complementarity, the distance between the Shine-Dalgarno sequence and the initiator codon, and the extent to which the Shine-Dalgarno sequence is masked by secondary structure. The length of the complementarity ranges from three to nine contiguous nucleotides (455). There is no experimental justification for the idea that messengers with longer-than-average Shine-Dalgarno sequences initiate with above-average efficiency. Nevertheless, it is clear from analysis of the ribosome-binding-site mutants just described that reducing the number of complementary nucleotides below a certain minimum impairs translation. A three-base-pair interaction often is adequate when the interaction involves the most favored region of 16S rRNA (i.e., the CUCC shown in boldface in Table 8), but the Shine-Dalgarno interaction sometimes involves nucleotides to the left or right of the CUCC sequence; in that less favorable situation, a three-base-pair complementarity allows only low-level expression (467). (Indeed, the exact site in 16S rRNA that participates in the Shine-Dalgarno reaction with a given messenger should probably be counted as a fourth parameter that modulates translational efficiency. One interesting mutant has been described in which translation was enhanced apparently by shifting the interaction from a less favorable region of 16S rRNA [AUCAC] to the more favorable site [CUCC] [69], but the generality of that finding remains to be established.)

The spacing between the Shine-Dalgarno sequence and the initiator codon is critical. On average, about seven nucleotides intervene between the 3' end of the purine run and the A of the AUG codon (465). Although some deviation from that value is tolerated, mutants in which the spacing was more than nine (mutant 102 in reference 467) or less than five (mutant zP18 in reference 433) nucleotides showed impaired translation. In the course of constructing recombinant plasmids which are able to express eucaryotic genes in *E. coli*, several investigators have systematically varied the distance between the Shine-Dalgarno site and the AUG codon (132, 189, 206, 378, 425, 488). The optimal distance was usually seven plus or minus two nucleotides, although in a few constructs a slightly wider spacing worked better. The importance of spacing can also be deduced from the observation that the functional initiator codon in bacterial and phage mRNAs is not always the first AUG or GUG triplet following the Shine-Dalgarno sequence. Three examples illustrate the point: the *E. coli trpC* initiation site (73), GAGGGuAAAuGAUG; the coliphage Qβ poly-

TABLE 8. Comparison of sequences at the 3' termini of small-subunit rRNAs

Source	Reference	Sequence[a]
Archaebacteria		
Halobacterium halobium	217	··· NNUCUGCGGCUGGAUCACCUCCU[3']
Eubacteria[b]		↓
E. coli	52, 431	··· AACCUGCGGUUGGAUCACCUCCUUA
B. stearothermophilus	446, 499	··· AAGGUGCGGCUGGAUCACCUCCUUUCUA
Chloroplasts		
Zea mays	413	··· AAGGUGCGGCUGGAUCACCUCCUUU
Euglena gracilis	340, 449	··· AAGGUGUGGCUGGAACAACUCCC
Mitochondria		
Yeasts	269	··· AACCUGCGGUGGGGCCUUAUAAAUCUUAAAUAUAUCUUACA
Wheat germ	406	··· AACCUGUGGCUGGAUUGAAUC(C)
Human[c]	90, 109	··· AAAGUGCACUUGGACGAAC
Bovine	11	··· AAAGUGUGUUGGAUAAAU
Cytoplasm of eucaryotic cells		
Mammals, frogs, silkworms, yeasts	166, 169, 273, 385, 394	··· AACCUGCGGAAGGAUCAUUA
Wheat germ	166	··· AACCUGCGGAAGGAUCAUUG

[a] Sequences are written in the 5'-to-3' direction (left to right), and are aligned by using the highly conserved GG marked by the arrow. Nucleotide modifications are not indicated.

[b] The sequence CUCC shown in boldface is the portion of *E. coli* 16S rRNA that most frequently participates in base pairing with mRNA (455).

[c] The major 12S rRNA from human mitochondria has the sequence shown. A minor species lacks the terminal one to three nucleotides. Both species are post-transcriptionally oligoadenylated at the 3' end (not shown)

merase initiation site (451), UAAGGA<u>UG</u>AA
<u>AUG</u>cAUG; and the *E. coli lacI* initiation site
(448), GGUG<u>GUG</u>A<u>AUG</u>UG. In each case, the
Shine-Dalgarno sequence and functional initia-
tor codon are shown in larger type, and the
underlined AUG and GUG triplets are nonfunc-
tional, presumably because they lie too close to
the Shine-Dalgarno site. I know of only one
example in which bacterial ribosomes appear
unable to determine which of two nearby AUG
triplets is the right initiator codon. In the se-
quence of coliphage fd gene 2, 4 nucleotides
separate the Shine-Dalgarno site from the first
AUG triplet, which functions despite the close
spacing, and 13 nucleotides separate the Shine-
Dalgarno site from the next AUG triplet, which
also functions (305). The observed ambiguity
might be rationalized on the grounds that neither
AUG is optimally positioned relative to the
Shine-Dalgarno sequence, although each falls
marginally within the acceptable range.

The third parameter that modulates mRNA
function is the degree to which the Shine-Dal-
garno sequence and initiator codon are seques-
tered by secondary structure. There are many
examples of genes (either natural mutants or
laboratory constructs) that are expressed poor-
ly, despite the presence of a polypurine tract in
an appropriate position for pairing with rRNA.
In each case, the mRNA is believed to assume a
conformation in which either the Shine-Dal-
garno site (170, 392) or the initiator codon (200)
or both (13, 132, 210) are sequestered. To what
extent may we generalize from these examples?
Is it true that in every efficiently utilized pro-
caryotic initiation site both the initiator codon
and the Shine-Dalgarno sequence are exposed?
The statement is true of many bacterial and
phage ribosome binding sites (414), but one can
think of a few apparent exceptions. In coliphage
T7 RNA species IIIb, both the AUG codon and
the Shine-Dalgarno sequence appear to be base
paired, judging from their resistance to nuclease
(382). The coat protein initiation site in coli-
phage MS2 RNA can also be drawn (on paper)
as a hairpin structure (308). Although the AUG
initiator codon of the coat cistron is accessible at
the top of the single-stranded loop, the presump-
tive Shine-Dalgarno sequence is sequestered
within the base-paired stem. At least two expla-
nations can be envisioned: (a) the conformation
of MS2 RNA is dynamic, and ribosomes bind to
the beginning of the coat protein cistron only
when that region assumes a more open confor-
mation, or (b) the functional Shine-Dalgarno site
for coat protein initiation is not the GGAG
sequence that lies just upstream from the AUG
triplet. Instead, the intricate secondary or tertia-
ry structure of MS2 RNA might bring the AUG

initiator codon of the coat cistron close to an
exposed purine-rich sequence that lies else-
where in the RNA chain. The notion that folding
of the mRNA might create a functional initiation
site from two nonadjacent regions has not been
critically tested. Two observations can be fitted
to hypothesis a or b: the isolated coat protein
initiation site (i.e., the ~30-nucleotide ribosome-
protected fragment) was shown to rebind to
ribosomes very inefficiently in vitro (453), and
the coat protein gene of coliphage MS2 was
poorly translated in vivo when it was separated
(via cloning into a plasmid) from the rest of the
phage genome (375).

(iii) **Is an exposed Shine-Dalgarno sequence,
appropriately positioned upstream from an ex-
posed initiator codon, sufficient to define a bacte-
rial ribosome binding site?** If an exposed Shine-
Dalgarno sequence, appropriately positioned
upstream from an exposed initiator codon, is
sufficient to define a bacterial ribosome binding
site, ribosomes should initiate at every site that
meets those criteria. Statistical analyses (144)
reveal that AUG triplets preceded by appropri-
ately spaced polypurine tracts occur randomly
throughout the *E. coli* genome—i.e., at the level
of primary structure, the motif is not unique to
initiation sites—but the degree to which the
nonfunctional sites are buried by secondary or
tertiary structure is unknown, and probably un-
knowable. A more promising approach to the
question is to look for direct evidence that other
features, in addition to the initiator codon and
Shine-Dalgarno sequence, contribute to recogni-
tion by procaryotic ribosomes. Biochemical and
genetic experiments provide a few such hints.
(a) Ribosome binding studies carried out with
synthetic AUG-containing oligonucleotides sug-
gest involvement of the nucleotide immediately
preceding the initiator codon (position -1) and
the nucleotide immediately following the initia-
tor codon (position $+4$). A pyrimidine in posi-
tion -1 promotes the highest level of oligonucle-
otide binding (102, 125), whereas a purine is
most effective in position $+4$ (403, 404). (The
latter effect was not observed in all experiments,
however [125].) A survey of bacterial and phage
ribosome binding site sequences revealed that
purines are indeed preferred in position $+4$, but
pyrimidines do not predominate in position -1
in natural mRNAs (465). The enhancing effect of
a purine, specifically adenosine, in position $+4$
has been shown most convincingly by mutagen-
izing that site. Taniguchi and Weissmann (483)
constructed mutants of coliphage Qβ in which
the coat protein initiator sequence was changed
from AUGG to AUGA. The mutant sequence
bound to ribosomes about three times more
efficiently than did the wild type. A popular,

although unproven, interpretation of those data is that the sequence AUGA forms a four-base-pair interaction with the anticodon loop in Met-tRNA. (b) In some cases, ribosome-protected mRNA fragments do not rebind efficiently to *E. coli* ribosomes (39, 453). In cases where the protected fragment (which was ~30 nucleotides long and included the initiator codon and Shine-Dalgarno sequence) failed to rebind to ribosomes, a slightly longer fragment of mRNA bound quite efficiently (39, 363). One interpretation is that outlying sequences provide an additional recognition signal for ribosomes. A simpler interpretation is that inclusion of outlying sequences alters the conformation of the RNA fragment, thereby exposing the initiator codon and Shine-Dalgarno site. (c) A third group of experiments that direct attention to features beyond the Shine-Dalgarno sequence involve mutations that inactivate ribosome binding sites. Such mutations have been identified upstream from the Shine-Dalgarno site (60, 116, 208a, 379), between the Shine-Dalgarno site and the initiator codon (132), and just beyond the start of the coding sequence (13). Again, two interpretations are possible, as in point b.

Other observations contradict the idea that features in addition to the Shine-Dalgarno sequence and the initiator codon participate (directly) in ribosome binding. There are many laboratory-constructed mutants in which the entire block of nucleotides upstream from the Shine-Dalgarno site has been deleted, with no deleterious consequences (307, 316; mutants 101 and 208 in reference 467). Many highly efficient chimeric genes have been created by cutting bacterial DNA right after a Shine-Dalgarno sequence and fusing it to a protein coding sequence from a eucaryotic source (162, 488). The ease of constructing functional chimeric ribosome binding sites constitutes compelling evidence against the idea that bacterial initiation sites require subtle features in addition to the initiator codon and an appropriately positioned Shine-Dalgarno sequence. Gold et al. (144) argue the opposite view, namely, that procaryotic ribosome binding sites contain other elusive but essential determinants. Their conviction is based on the lack of correlation between translational efficiency and the strength of the Shine-Dalgarno interaction, but that view ignores the role of mRNA conformation in regulating ribosome access to potential initiation sites.

Can the Shine-Dalgarno mechanism be extrapolated to other systems? In addressing the question of whether the Shine-Dalgarno mechanism can be extrapolated to other systems, a useful first step is to compare the 3'-terminal sequences of small-subunit rRNAs from various sources. As shown in Table 8 and mentioned previously, the 3'-proximal portion of 16S–18S rRNA is highly conserved, but the homology stops just short of the 3' terminus. Thus, the critical sequence CCUCC in *E. coli* 16S rRNA is absent from eucaryotic cytoplasmic 18S rRNA and from the small-subunit rRNAs of mitochondrial origin. The sequence CCUCC (or something very close) is present, however, in 16S rRNA from chloroplasts, as well as in the primitive archaebacteria.

It seems likely that chloroplast ribosomes mimic the bacterial initiation mechanism. Although a complex between mRNA and 16S rRNA has not yet been demonstrated directly, the sequence of the ribulose bisphosphate carboxylase gene from chloroplasts includes a perfectly positioned GGAGG sequence just upstream from the AUG initiator codon (297, 554). A similar sequence occurs in a few other chloroplast genes that have been studied (247). Moreover, Steege et al. (449) found that chloroplast ribosomes behave similarly to *E. coli* ribosomes in their ability to selectively bind purine-rich oligonucleotides that are complementary to the 3' terminus of small-subunit rRNA. It is intriguing that those experiments worked even with ribosomes from *Euglena gracilis* chloroplasts, in which the 3'-terminal sequence of rRNA is similar, but not identical, to *E. coli* (see Table 8). Thus, the Shine-Dalgarno mechanism may have been retained in chloroplasts without strictly conserving the sequence!

The archaebacteria, in contrast, seem to have conserved the Shine-Dalgarno sequence at the 3' end of 16S rRNA without retaining the mechanism that goes with it. That surprising conclusion emerged when Dunn et al. (99) cloned and sequenced the bacteriorhodopsin gene from *Halobacterium halobium*. The mRNA encoding that protein has only three nucleotides upstream from the AUG initiator codon. Although mRNAs from other archaebacterial genes must be analyzed before we can generalize, the first hint from *H. halobium* is that mRNA · rRNA complementarity does not play a role in initiation.

Since experiments directly probing the initiation mechanism have not been attempted with mitochondrial mRNAs and ribosomes, nucleotide sequences provide our only clues. In mammalian mitochondrial mRNAs, the initiator codon occurs either directly at, or a few nucleotides down from, the 5' terminus. (See The Structure of Messenger RNA.) Thus, there is no opportunity for mRNA · rRNA base pairing. Indeed, the 3' end of 12S rRNA from mammalian mitochondria lacks all vestiges of the CCUCC sequence found in *E. coli*. But

mRNAs from yeast mitochondria are surprisingly different from mammalian mitochondrial mRNAs: yeast mitochondrial mRNAs do have a 5' untranslated sequence, often of extraordinary length. The 3'-terminal sequence of small-subunit rRNA from yeast mitochondria is also unique, as shown in Table 8. Thus, it does not seem unreasonable to postulate that yeast and mammalian mitochondrial ribosomes bind to mRNA via different mechanisms. Li et al. (269) have proposed that the sequence AUAUCUUAAA near the 3' end of yeast mitochondrial 15S rRNA pairs with a complementary sequence (4 to 10 nucleotides long) present in the 5' leaders of at least four, and possibly six, mitochondrial mRNAs, but the location of the complementary sequence relative to the initiator codon varies tremendously—from 6 to 107 nucleotides. Given the unusually long, unusually AU-rich leader sequences on yeast mitochondrial mRNAs, there is a high probability that a sequence complementary to (some portion of) AUAUCUUAAA will fortuitously occur somewhere within the 5' leader segment. That does not constitute compelling evidence for a Shine-Dalgarno interaction.

The possibility of mRNA · rRNA base pairing in eucaryotes is difficult to assess. Since the CCUCC sequence found near the 3' end of bacterial 16S rRNA is absent from eucaryotic 18S rRNA (166), the hypothetical Shine-Dalgarno interaction would have to involve some other sequence in 18S rRNA. One might hope to identify that sequence by incubating eucaryotic ribosomes with a mixture of ^{32}P-labeled oligonucleotides and asking which oligonucleotides bind. The answer is that none show the type of specific binding observed with procaryotic ribosomes (449, 455)! In a different approach, Nakashima et al. (321) used a psoralen derivative to cross-link mRNA to 18S rRNA after forming initiation complexes with wheat germ ribosomes, but they obtained cross-linking even with poly(U) and other templates that are not complementary to any sequence near the 3' end of 18S rRNA. Thus, their experiment neither proves that a Shine-Dalgarno interaction occurs in eucaryotes nor tells us where in the rRNA to look for such a reaction. The most popular recourse has been to survey eucaryotic mRNAs for complementarity to any portion of the 3'-terminal sequence of 18S rRNA. Thus, the eucaryotic equivalent of a Shine-Dalgarno mechanism has been proposed to involve nucleotides 2 to 8 (549), 6 to 10 (35, 142), 10 to 17 (319), and 18 to 24 (255). The presence of a stable hairpin structure near the 3' end of 18S rRNA (involving nucleotides 10 to 18 and 23 to 31) probably precludes some of the postulated interactions

with mRNA. Sargan et al. (398) have proposed an alternative model which at least is compatible with the secondary structure of 18S rRNA. They postulate that nucleotides flanking the base of the hairpin structure at the 3' end of 18S rRNA interact with the semiconserved sequence CCACC that precedes the initiator codon in many eucaryotic mRNAs. That intriguing suggestion merits further study. Many of the proposed mRNA-rRNA interactions in eucaryotes involve only patchy complementarity (168, 255, 285, 319, 549). Such interrupted complementarity is of questionable significance, since procaryotic Shine-Dalgarno sequences of proven function always involve contiguous nucleotides. Proponents of the Shine-Dalgarno mechanism in eucaryotes also tend to accept complementary sequences that occur anywhere within the long 5' untranslated region of the messenger. Even with these (too) liberal criteria, many eucaryotic mRNAs lack significant potential for pairing with 18S rRNA (86). Nevertheless, one might entertain the hypothesis that mRNA · rRNA pairing facilitates ribosome binding in those few eucaryotic mRNAs that do have a long complementary sequence (17, 124, 166, 255, 284, 552). In one case where the putative Shine-Dalgarno site was experimentally deleted from an essential adenovirus gene, viral replication and transformation were not impaired (341). Before concluding that the putative Shine-Dalgarno sequence plays no role, however, it will be necessary to quantitate the yield of protein from the wild-type and mutant viral mRNAs. Yamaguchi et al. (537) recently attempted to correlate the translational efficiency of two plant virus mRNAs with the presence or absence of a sequence complementary to the 3' end of 18S rRNA, but they presented no evidence that the AUG triplet on which they focused is the functional initiator codon in cucumber mosaic virus RNA-5. Thus, the case for a Shine-Dalgarno interaction in eucaryotes is not strong.

Eucaryotic Ribosomes Probably Initiate via a Scanning Mechanism

A considerable body of circumstantial evidence (reviewed in references 237, 241, 242, and 245) supports the hypothesis that 40S ribosomal subunits bind initially at or near the 5' end of the mRNA and then migrate down to the AUG initiator codon. In the simplest version of this "scanning model," the 40S ribosomal subunit (with associated initiation factors, of course) would advance toward the interior of the messenger until it encountered the first AUG triplet, at which point a 60S subunit would join and the first peptide bond would form. That version of the model says, in effect, that the functional

initiator codon is defined merely by its position—i.e., closest to the 5' end of the mRNA. However, the current catalog of published eucaryotic sequences, totaling more than 200 mRNA species, includes 18 messengers in which ribosomes initiate at an AUG triplet that is not first in line (15 of these are tabulated in reference 245; the others are described in references 75, 177, and 543). In those 18 exceptional mRNAs, one or more AUG triplets occur upstream from the beginning of the protein coding sequence. Inspection of the nucleotide sequences flanking the apparently nonfunctional upstream AUG triplets led to a modified version of the scanning model (243). The current working hypothesis is that 40S ribosomes enter at the 5' end of the mRNA and advance toward the interior, searching for an AUG codon, but the efficiency with which a 40S ribosomal subunit recognizes an AUG triplet (and stops migrating) depends on the flanking sequences. ANNAUGG has been tentatively identified as the optimal context for initiation by eucaryotic ribosomes. That identification is based on a survey of nearly 200 mRNA sequences (see Table 6) as well as binding experiments carried out in vitro with various AUG-containing oligonucleotides (243). According to the modified scanning model, if the first AUG codon encountered by the migrating 40S ribosome occurs in the optimal sequence context, 40S subunits will initiate uniquely at that site; however, if the first AUG triplet occurs in a less favorable context (e.g., GNNAUGU or UNNAUGG), some 40S ribosomal subunits will stop and initiate there, whereas some will bypass that site and initiate farther downstream. This introduces an interesting flexibility into the system. A single mRNA can direct synthesis of two proteins if the first AUG triplet occurs in a suboptimal sequence context (thereby allowing some ribosomes to advance to the next potential initiator codon) and both the first and the second AUG triplets are followed by open reading frames. Indeed, a few eucaryotic mRNAs that conform to this pattern and that direct synthesis of two proteins have been found. These are the messengers listed in Fig. 3C. But bifunctional mRNAs are rare in eucaryotes. Normally, the 5'-proximal AUG triplet occurs in a favorable sequence context; ribosomes initiate only at that site, and the messenger directs synthesis of only one protein. Thus, the scanning model rationalizes the monocistronic character of most eucaryotic mRNAs. The scanning mechanism also rationalizes the ease with which internal initiation sites are activated by cleaving the mRNA (see Table 7). Cleavage creates new ends, which serve as entry sites for 40S subunits. Circularizing the

messenger understandably has the opposite effect; i.e., eliminating the ends of the mRNA abolishes the ability of eucaryotic ribosomes to bind (Table 7). The scanning model postulates that entry of ribosomes at the 5' end of the mRNA is a step distinct from recognition of the AUG initiator codon. Thus, it is not surprising that the m^{7}G cap facilitates translation even with messengers in which the AUG initiator codon lies hundreds of nucleotides downstream from the 5' terminus (22, 220).

Translational Control at the mRNA Level

Alternative forms of mRNA. It is not uncommon to find in bacterial cells multiple forms of mRNA derived from a single gene. mRNAs differing in primary structure can be generated by varying the sites where transcription begins and ends or by varying the extent of posttranscriptional processing (21, 223, 392). These parameters sometimes show an interesting interdependence; i.e., the extent of processing may depend on where transcription begins and ends (151). The chief culprit in processing of bacterial and bacteriophage mRNAs is ribonuclease III. Cleavage (outside of the protein coding sequence) by that enzyme occasionally converts an inactive precursor to a functional message (97) and occasionally inactivates a message (151), but most often it has no discernible effect on the translational capacity of the transcript (97, 138, 165). The rIIB protein of coliphage T4, for example, can be translated from either a polycistronic mRNA or a ribonuclease III-generated monocistronic mRNA. The two forms of mRNA do not function identically, however, since certain mutations in or around the ribosome binding site reduce translation from only the monocistronic form (433). There are a few examples from bacterial and bacteriophage systems where transcripts initiated at either of two promoters differ markedly in their ability to be translated (368, 369). Such observations can sometimes be rationalized in terms of the Shine-Dalgarno sequence requirement (368), although that explanation fails in other cases (369). An additional source of variability in procaryotic mRNAs is the ability to assume alternative secondary structures—sometimes with profound consequences for translation. The mechanism of erythromycin-induced resistance in *Staphylococcus aureus* illustrates the intricate control that can be achieved via changes in mRNA conformation (187).

In eucaryotes, initiation of transcription at multiple sites is a common occurrence (28, 79, 112, 157, 549). In the examples cited, selection of the transcriptional start site appears to be imprecise: transcripts are initiated at several

sites within a span of 10 to 50 nucleotides, resulting in a heterogeneous mRNA population. A few eucaryotic genes produce alternative forms of mRNA via a more systematic mechanism; i.e., one promoter or another is active, depending on circumstances within the cell. During the course of infection by simian virus 40, for example, the transcriptional start site for the T-antigen gene shifts from around position 5230 to a position approximately 40 base pairs upstream (135). Promoter switching also occurs during the course of adenovirus infection (72a). Alternative promoters exist for a few cellular genes, including mouse α-amylase (545), rat calcitonin (6), and yeast invertase (62). Only in the last case have the alternative transcripts been shown to function differently; i.e., ribosomes initiate at a different AUG codon in each form of invertase mRNA, in a manner predicted by the scanning model. The result is that one mRNA species directs synthesis of a secreted form of invertase, whereas the other encodes a truncated intracellular version of the enzyme (62). Multiple forms of mRNA can also be produced from one gene by varying the splicing pattern. Changing the site of splicing within the protein coding region obviously has drastic effects on the structure of the encoded protein. The consequences of varying the splicing pattern within the 5' untranslated region are less obvious, although such variation is sometimes observed (94, 251, 493). The rat insulin-II gene provides an interesting example of translational regulation at the level of mRNA structure. In contrast with the functional insulin-II mRNA that is produced in normal pancreatic tissue, a certain β-cell tumor produces a nonfunctional form of insulin-II mRNA that is initiated and spliced normally, but is defective at the 5' end; most likely, it lacks the m^7G cap (77a). Finally, some eucaryotic cellular genes have more than one potential site for poly(A) addition, thus yielding a mixed population of mRNAs that differ in the lengths of the 3' noncoding regions (399, 417, 490). Although no functional differences among the resulting mRNA forms have yet been detected, the available in vitro translation systems might not be adequate to reveal subtle variations in translational efficiency or not-so-subtle differences in mRNA stability.

mRNA binding proteins. In procaryotic systems, there are several examples of proteins that bind in a highly specific fashion to one (or a few) mRNAs, thereby preventing translation. The list of translational repressor proteins includes the coat protein and polymerase of coliphage MS2 (245), the single-stranded-DNA binding protein encoded by coliphage T4 gene 32 (265), several of the ribosomal proteins from E. coli (335), gene V protein of coliphage f1 (311, 541), E. coli RNA

polymerase (258a, 349), and possibly the regA protein of phage T4 (221). In the first three examples mentioned, the target site for the repressor protein has been mapped to a region near the AUG initiator codon (32, 248, 250, 521), thus rationalizing the inhibitory effect of the protein on translation. Ribosomal proteins L10 and L12, however, seem to inhibit by binding some distance upstream from the initiator codon (208a, 540). (Although the target sequence of the regA protein maps near the ribosome binding site [221], it is not known whether regA acts at that site to inhibit translation or to promote mRNA degradation.) Several of the repressor proteins are autoregulatory; i.e., the protein arrests translation by binding to its own mRNA. Most, if not all, of the procaryotic translational repressors serve another major function in addition to their role in regulating translation.

No specific translational repressor proteins of the sort described above have yet been found in eucaryotes. There are intriguing hints that Drosophila heat shock proteins might inhibit their own translation (86a; S. Lindquist, personal communication) and that procollagen-derived peptides selectively inhibit collagen synthesis (188), but those stories await further study. Eucaryotic mRNAs are closely associated with a rather large set of proteins in vivo, forming the familiar ribonucleoprotein particles. The protein components of ribonucleoproteins are nonspecific in that they do not seem to pick and choose among mRNAs, but the proteins are specific for mRNA as opposed to other intracellular RNAs (415). There are hints from in vitro experiments that the protein components of ribonucleoproteins mediate both positive (402) and negative effects on translation (30). The only accessory proteins identified so far that have a well-defined role in regulating eucaryotic translation are the components of the signal recognition particle, which temporarily arrests elongation of nascent secretory polypeptides (517). However, the target for binding of the signal recognition particle is not the mRNA but rather the NH_2-terminal amino acid sequence of the nascent polypeptide! The translational arrest is released when the complex binds to microsomal membranes (304, 517). One other group of proteins associated with eucaryotic RNAs has provoked speculation about a possible regulatory role in translation. I refer to proteins that are covalently linked to the 5' termini of some viral genomic RNAs. There is good evidence that the 5'-terminal protein functions in initiating replication of the viral genome (529). The possibility that the protein also serves a negative role—namely, occluding the binding of ribosomes—has been postulated (242), but the available evidence suggests otherwise (146). Neverthe-

less, it is intriguing that uninfected cells contain an enzyme that cleaves the 5'-terminal protein from viral RNAs (7). One is thus led to wonder whether some cellular RNAs also carry 5'-linked proteins.

CONCLUSIONS AND PERSPECTIVES

The tremendous amount of data accumulated within the past 15 years has revealed much about the initial steps in protein synthesis; it goes without saying that much remains to be learned. The Shine-Dalgarno mechanism goes far toward explaining how procaryotic ribosomes select initiation sites in mRNA, particularly if one takes into account the modulating effects of mRNA conformation. Unfortunately, RNA secondary structures are easier to draw on paper than to verify experimentally. Thus, it is difficult to prove rigorously the simple view (which I favor) that an exposed Shine-Dalgarno site preceding an exposed AUG codon is sufficient to define a procaryotic ribosome binding site.

The anonymous writer of a *Nature* News and Views column (vol. 226, 16 May 1970, p. 592) wrote that "molecular biologists who have started to elucidate the mechanism of protein synthesis in nucleated cells are in the envious or invidious position of *knowing what the results of their experiments should be* because they already know in detail how bacteria make proteins" (emphasis added). The results of experiments with eucaryotic mRNAs and ribosomes have often turned out to be other than they should be. The observation that translation is restricted to the 5' end of eucaryotic mRNAs provided the first clue that the initiation mechanism in eucaryotes differs from the procaryotic paradigm. The modified scanning model offers a tenable description of how eucaryotic ribosomes arrive at the correct initiation site, but the current formulation of the scanning hypothesis is undoubtedly oversimplified. It does not fully explain the differences in translational efficiency among various mRNAs, for example. Although the 5'-terminal cap enormously enhances translation of those mRNAs that have a cap, the mechanism of that enhancement is not understood, nor is there an explanation (except by untested hypothesis) of how a few viral mRNAs get by so well without a cap.

The overwhelming structural similarities between bacterial and chloroplast ribosomes lead one to expect functional analogies between those systems. Indeed, the first sequences deduced for chloroplast messengers support the possibility of mRNA · rRNA base pairing. The available information from mitochondrial systems is insufficient to reveal the workings of the initiation process, but we can speculate. The structural differences between mRNAs from mammalian and yeast mitochondria raise the interesting possibility that disparate initiation mechanisms operate in mitochondria from different sources. The 5'-end-dependent mechanism postulated for eucaryotic cytoplasmic ribosomes might also work with mammalian mitochondrial ribosomes, as suggested by Montoya et al. (314). Since the AUN initiator codon is so close to the 5' terminus of mammalian mitochondrial mRNAs, little additional information would be needed to define the initiation site. But binding at the 5' terminus, followed by scanning, does not seem compatible with the structure of yeast mitochondrial mRNAs, where the very long 5' leader sequence often contains AUG triplets upstream from the functional initiation site. Neither does the notion of mRNA · rRNA base pairing seem to be supported by the available sequence data from yeast mitochondrial mRNAs. I suspect that a novel initiation mechanism is at work in that system. That, and other surprises, will undoubtedly emerge from future studies on the initiation of protein synthesis.

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